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# CANCER RESEARCH

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CANCER RESEARCH

A MONTHLY JOURNAL OF ARTICLES AND ABSTRACTS REPORTING CANCER RESEARCH

VOLUME 8 APRIL, 1948 NUMBER 4

# The Action of Mustard Gas ($\beta\beta'$Dichlorodiethylsulphide) on Living Cells *in Vitro*

## I. The Immediate Cytological Effects of Mustard Gas and of Its Hydrolysis Products

## II. The Effect on Cell Growth of Adding Small Concentrations of Mustard Gas to the Culture Medium

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(Received for publication October 21, 1947)

### INTRODUCTION

Tissue cultures are ideal material on which to study the direct effects of toxic agents on living cells. The observations described in Part I concern the immediate effects of mustard gas, as liquid and as a vapor, and of its hydrolysis products when directly applied to living chick fibroblasts growing *in vitro;* Part II deals with the effect on cell-growth and survival, of adding low concentrations of mustard gas to the culture medium.

The main object of the investigation was to discover how mustard gas enters and kills cells, and whether it is mustard gas itself or some identifiable product which is the active agent. Experiments were also made to find (a) the minimal (immediate) lethal concentration of mustard gas for living fibroblasts, (b) whether incubation in plasma detoxicates mustard gas, and (c) the effect on cell-survival of continued cultivation in concentrations of the agent which are not immediately lethal. The work was done during the war for the Department of Chemical Defence Research and Development of the Ministry of Supply; recently the biological action of mustard gas has acquired a more general interest owing to the increasing clinical use of "mustard" compounds in the treatment of cancer (13).

The results were originally recorded in a series of reports made to the Ministry of Supply between 1939 and 1943 (1, 5-10), and we are indebted to the Chief Scientist of the Ministry of Supply for permission to publish them. One of us (C. B. A.) was in receipt of personal grants from the Medical Research Council during the period.

The "mustard gas" used in all the experiments was pure, re-distilled, $\beta\beta'$-dichlorodiethyl sulphide.

## I. The Immediate Cytological Effects of Mustard Gas and of Its Hydrolysis Products

### TECHNIC

#### Tissue Culture

Small pieces of choroid and sclerotic from 10 to 12 day chick embryos were grown by the hanging drop method on 1¼ inch square coverslips in a mixture of equal parts of fowl plasma and chick embryo extract. The cultures were used either 2 days after explantation or 2 days after the first subculture.

#### Cytology

All the fixed and stained preparations were mounted whole. For general histological examination the cultures were fixed in 5 per cent acetic alcohol, stained with Ehrlich's hematoxylin, dehydrated, cleared in xylol and mounted in Canada balsam. To show cytoplasmic details they were fixed in 2 per cent osmic acid followed by Zenker's fluid without acetic acid, stained with Ehrlich's or Heidenhain's iron hematoxylin and mounted in

NOTE: Because of accelerated production schedule, the authors have not read proof of this paper.

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Gurr's aqueous mounting medium. For the study of nuclear structures, cultures were fixed in Susa and stained by Feulgen's method.

A histochemical technic was devised to demonstrate the presence and distribution of mustard gas in the cells of treated cultures (6). It was based on the reaction between dichlordiethyl sulphide and selenium dioxide in strong acid; at room temperature mustard gas reacts slowly to give an orange-colored solution of colloidal selenium, thiodiglycol reacts more quickly but gives a chocolate-colored precipitate, presumably of the element, whilst dichlordiethyl sulphone and divinyl sulphone, which are possible oxidation products of the sulphide, produce no color change. Tissue cultures contaminated with mustard gas stain orange when treated with $SeO_2$ at room temperature whereas untreated controls remain colorless. The contaminated cultures were either inverted over a hollow-ground slide filled with a 1 per cent solution of $SeO_2$ in 50 per cent $H_2SO_4$ or placed in a bath of this reagent; the former method enabled the effect of the $SeO_2$ to be observed under the microscope. After not more than 30 minutes' contact with the solution at room temperature, the cultures were washed with water or 50 per cent alcohol, lightly stained with Ehrlich's hematoxylin, dehydrated, cleared in xylol and mounted in Canada balsam. Such preparations were not permanent as much of the selenium appeared to be dissolved out by the xylol of the Canada balsam.

## EXPERIMENTS

### I. The Effect of Liquid Mustard Gas

1. *The effect on living cells* (5, 7).—Forty-two cultures were treated. The coverslip of each was removed from the hollow-ground slide and placed in contact with a drop of liquid mustard gas on a flat slide, where it was supported by two thin strips of paraffin wax painted across the glass. The preparation was then sealed with molten paraffin wax.

In the normal living culture the fibroblasts forming the zone of outgrowth (Plate I, Fig. 1) are very thin and transparent and usually triangular or fan-shaped; the broad end, which generally points away from the explant, is prolonged into spiky or branched pseudopodia. The oval nucleus containing two or more irregular nucleoli lies in the narrow part of the cell and the ground substance of both nucleus and cytoplasm appears homogeneous. The most conspicuous objects in the cytoplasm are the groups of small, refractile fat globules (Fig. 1). By ordinary direct illumination the mitochondrial filaments are almost invisible in the living cell.

When such a culture is placed in contact with mustard gas and immediately examined under a microscope in a hot box, the first visible change in the cells is a swelling of the fat globules. Within a few seconds this is followed by the coagulation and death of the cell: the outlines of both cytoplasm and nucleus suddenly become bold and distinct and a reticular coagulum appears in the previously clear protoplasm. There is very little distortion of the shape of the cell or of its nucleus, and the effect of liquid mustard gas closely resembles that of some rather drastic histological fixative such as Zenker's fluid or formalin.

The fat vacuoles continue to swell rapidly and as they enlarge many coalesce so that after 10 to 30 minutes the cytoplasm is dilated by a relatively small number of very big globules (Figs. 1 and 2). The enlargement of the fat vacuoles then slows down and at the same time much finer globules appear in the intervacuolar protoplasm. Some of these seem to form in the nucleus also, but it is difficult to be sure whether they are in the nucleus itself or merely in the film of cytoplasm above and below it. These fine droplets multiply and enlarge until the whole cell is infiltrated with refractile material. Sixteen control cultures treated with olive oil or cod liver oil showed neither swelling of the fat vacuoles nor infiltration of the cells with

## DESCRIPTION OF PLATES

The photomicrographs were made by Mr. V. C. Norfield, Head Assistant at the Strangeways Research Laboratory.

*Abbreviations*

f. g fat globules N. nucleolus
f. v. fat globules nu. nucleus
m. mitochondria p. precipitate

### DESCRIPTION OF FIGURES 1 TO 5

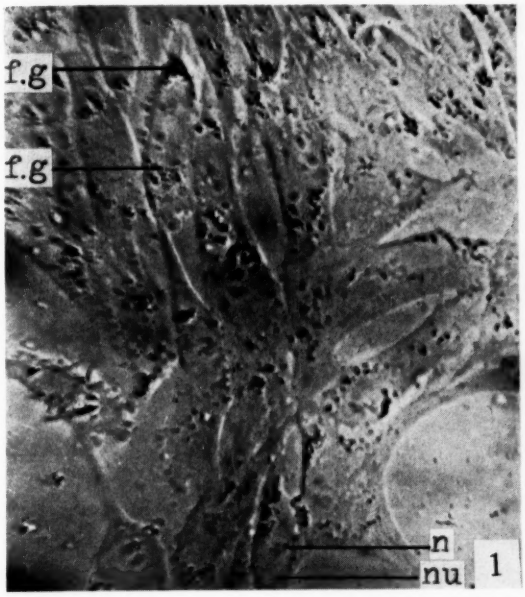
Fig. 1.—Part of the zone of outgrowth of a living culture of fibroblasts after 48 hours' growth. The fat globules, nucleus and nucleoli can be seen. Mag. × 400.

Fig. 2.—The same cells 30 minutes after the application of liquid mustard gas. Note the great enlargement of the fat globules. Mag. × 400.

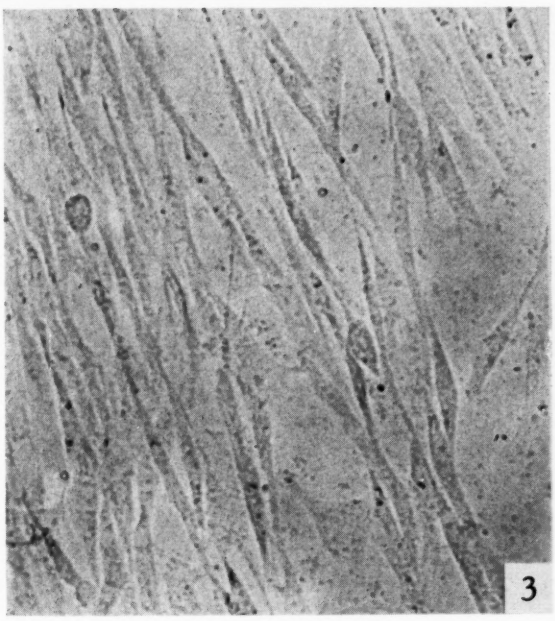
Fig. 3.—Part of the zone of outgrowth of a living (control) culture after 48 hours' growth. Mag. × 250.

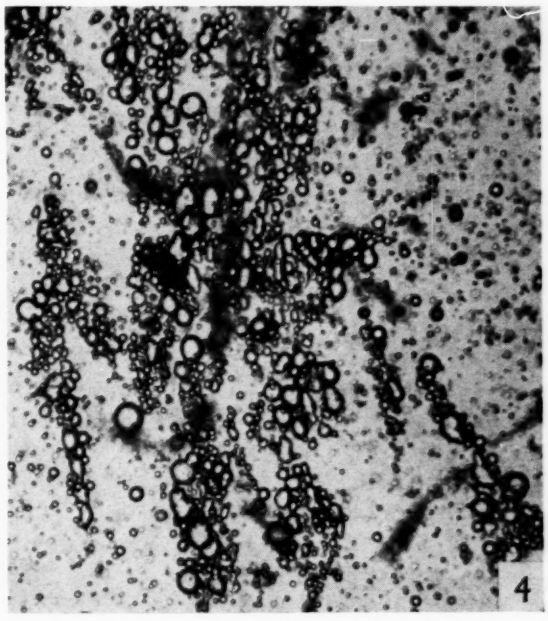
Fig. 4.—Sister culture to that shown in Fig. 3, after fixation in formalin and 2 hours' treatment with liquid mustard gas. Note the enormous engorgement of the cells with refractile droplets of mustard gas. Mag. × 250.

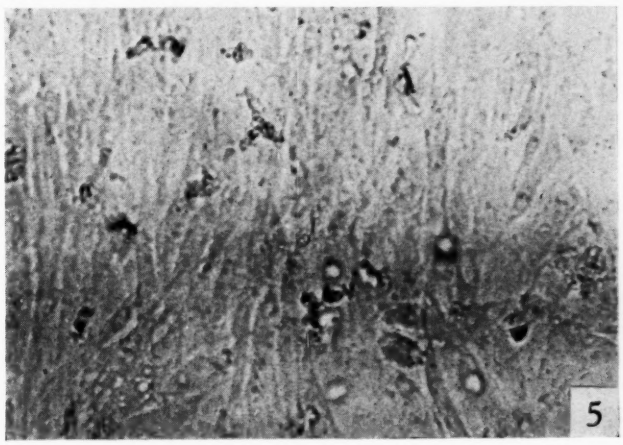
Fig. 5.—Sister culture fixed in 10 per cent formalin, extracted with alcohol and ether and then exposed to liquid mustard gas for 2 hours. No refractile droplets have appeared in the cells. Mag. × 250.








Figs. 1–5

droplets, but both these phenomena have been described in cultures treated with liquid lewisite (11).

To find whether the globules which form in the cells are dichlordiethyl sulphide, cultures previously treated with mustard gas were inverted over a hollow-ground slide filled with selenium dioxide solution and watched under a microscope. Within a few minutes one or more small, dark orange granules appeared inside each refractile droplet and simultaneously the whole globule gradually turned yellow, thus giving the typical mustard gas reaction (Fig. 16). The central explant and the denser parts of the plasma clot also became bright yellow. Control cultures not previously treated with mustard gas showed none of these changes.

In cultures exposed to liquid mustard gas and then fixed with acetic alcohol and stained with Ehrlich's hematoxylin, the cells differ from those of normal cultures fixed and stained in the same way mainly in the much larger size of their fat vacuoles. The more critical fixation with osmic acid and staining with iron hematoxylin, however, reveal many other important differences between the treated and normal cells (Figs. 6 and 7). In the former the ground substance of nucleus and cytoplasm is coarsely reticular; the treated nuclei are bounded by a distinct black line and the mitochondria are reduced to faintly stained, irregular streaks and blobs which are difficult to distinguish; in the latter the ground substance of the protoplasm appears almost homogeneous, the nucleus is not bounded by a dark line and the mitochondria are beautifully clear, long, black filaments.

The changes produced by mustard gas in the fat globules are still visible in the cultures after fixation with osmic acid. When the treated cells are fixed while the fat vacuoles are beginning to swell, the outline of the vacuoles is irregular, owing to the extraction of the mustard gas content by the histological treatment, and the osmicated substance is diffuse and greyish in color instead of greenish black. In vacuoles fixed during the later stages of enlargement, the osmicated fat is reduced to a crumpled surface membrane and finally disappears altogether (Fig. 7).

The internal structure of the nucleus as demonstrated by the Feulgen method (Figs. 13 and 14) is remarkably well preserved in the dead cells even after 2½ hours' contact with liquid mustard gas. In the deeper parts of the clot, where the agent had penetrated more slowly, the nuclei are slightly shrunken and pycnotic, but elsewhere no important difference can be detected between the normal and treated nuclei. The mitotic figures also are well preserved; in anaphase and metaphase the chromosomes are rather more closely clumped than in the controls, but they stain normally and even the delicate threads of prophase and late telophase appear almost unaltered (Figs. 13 and 14).

2. *The effect of protoplasmic coagulation and of fat extraction on the intracellular penetration and accumulation of liquid mustard gas.*—Experiments were made (8) to find whether liquid mustard gas would enter coagulated cells in the same way as it enters living cells,[1] and if so whether its entry would be affected by the extraction of fats and lipoids from the protoplasm. The experimental procedure is summarized in Table I.

When cultures coagulated by either formalin or heat and not extracted with alcohol and ether are placed in contact with liquid mustard gas and observed under the microscope at body temperature, the fat globules begin to swell though more slowly than in living cultures treated with the agent, possibly because the cells are slightly screened by the coagulated culture medium. Eventually the fat vacuoles become enormously distended (Figs. 3 and 4), smaller droplets appear between these, and finally the cells are bloated and engorged with refractile material. Treatment with $SeO_2$ produces coarse granules of orange or brown precipitate in the cytoplasmic droplets; control cultures fixed in formalin and not contaminated with mustard gas give no reaction.

A very different result is obtained when liquid mustard gas is applied to cultures extracted with alcohol and ether after coagulation by formalin or heat. No refractile droplets appear in the protoplasm (Fig. 5) and the cells either give no reaction with $SeO_2$ or acquire a fine, scanty precipitate in contrast to the coarse granules formed in the unextracted tissue. The explant and the less expanded cells near it, however, stain bright orange and usually contain some granular precipitate though much less than in the unextracted cultures; whether this reaction is due to a small quantity of mustard gas evenly distributed through the cell or merely to thiodiglycol is not known.

These results show that while coagulation of the tissue has no significant effect on the entry of mustard gas into the cells, the dense engorgement of the protoplasm with the agent depends on the cellular fat content.

II. The Effect of Saturated Gas Vapor (6, 7)

To study the effect of saturated mustard gas vapor on living cells, the coverslip of a normal hanging drop culture was unsealed and raised, a small drop of the liquid agent was deposited in the

[1] We are indebted to Professor S. Sugden, F.R.S. for this suggestion.

hollow of the slide and the coverslip was replaced and resealed. As 37° C., in the small space (approx. 3 ml.) below the culture, equilibrium between liquid and vapor is rapidly attained. Thirty-three cultures were treated.

Like liquid mustard gas the saturated vapor almost immediately coagulates the protoplasm without greatly distorting the form of the cell, but though there is some coalescence and enlargement of the fat vacuoles the cells do not become infiltrated with refractile droplets. After 2½ hours' exposure to the vapor followed by treatment with $SeO_2$, the explant becomes bright orange and the cells in the zone of outgrowth contain orange precipitate (Fig. 17), showing that the vapor enters the cells in the same way as the liquid but to a lesser extent.

After fixation with osmic acid and staining with

TABLE I: SUMMARY OF EXPERIMENTS ON THE EFFECT OF PROTOPLASMIC COAGULATION AND OF FAT EXTRACTION ON THE INTRACELLULAR PENETRATION AND ACCUMULATION OF MUSTARD GAS

EXPERIMENT 1

| No. of set | No. of cultures | Treatment |
|---|---|---|
| 1 | 6 | (1) 10% Formol: 10 mins.<br>(2) Rinsed distilled water<br>(3) Saline (Pannett & Compton's)<br>(4) Mustard gas: 2 hours<br>(5) 1% Selenium dioxide solution: 30 mins. |
| 2 | 6 | (1) 10% Formol: 10 mins.<br>(2) Rinsed<br>(3) Absolute alcohol: 30 mins.<br>(4) Ether: 30 mins.<br>(5) Hydrated (absolute alcohol: 50% alcohol; water)<br>(6) Saline<br>(7) Mustard gas: 2 hours<br>(8) $SeO_2$: 30 mins. |
| 3 | 6 | (1) Coagulated by steam<br>(2) Saline<br>(3) Mustard gas: 2 hours<br>(4) $SeO_2$: 30 mins. |
| 4 | 6 | (1) Coagulated by steam<br>(2) Absolute alcohol: 30 mins.<br>(3) Ether: 30 mins.<br>(4) Hydrated<br>(5) Mustard gas: 2 hours<br>(6) $SeO_2$: 30 mins. |
| 5 | 3 | (1) Saline<br>(2) Mustard gas: 2 hours<br>(3) $SeO_2$: 30 mins. |

EXPERIMENT 2

| No. of set | No. of cultures | Treatment |
|---|---|---|
| 1 | 6 | (1) 10% Formol: 5 mins.<br>(2) Rinsed<br>(3) Absolute alcohol: 25 mins.<br>(4) Ether: 5 mins.<br>(5) Hydrated<br>(6) Saline<br>(7) Mustard gas: 2 hours<br>(8) $SeO_2$: 30 mins. |
| 2 | 6 | (1) 10% Formol: 5 mins.<br>(2) Rinsed<br>(3) Absolute alcohol: 25 mins.<br>(4) Ether: 5 mins.<br>(5) Hydrated<br>(6) $SeO_2$: 30 mins. |
| 3 | 6 | (1) 10% Formol: 5 mins.<br>(2) Rinsed<br>(3) Saline<br>(4) Mustard gas: 2 hours<br>(5) $SeO_2$: 30 mins. |
| 4 | 6 | (1) 10% Formol: 5 mins.<br>(2) Rinsed<br>(3) Saline<br>(4) $SeO_2$: 30 mins. |

## DESCRIPTION OF FIGURES 6 TO 12

All the photographs in this plate are of cultures fixed in osmic acid and stained with iron hematoxylin.

FIG. 6.—Normal fibroblasts in a 48 hour culture showing nuclei, nucleoli, fat globules and mitochondrial filaments. The ground substance of nucleus and cytoplasm appears fairly homogeneous. Mag. × 1,450.

FIG. 7.—Similar cells after 2½ hours' contact with liquid mustard gas. The shape of the cells is well preserved but they differ from normal fibroblasts (Fig. 6) in the coarse reticular structure of their protoplasm, the dark outline of the nucleus, the enormous size of the fat vacuoles and the disintegration of the mitochondria. Mag. × 1,450.

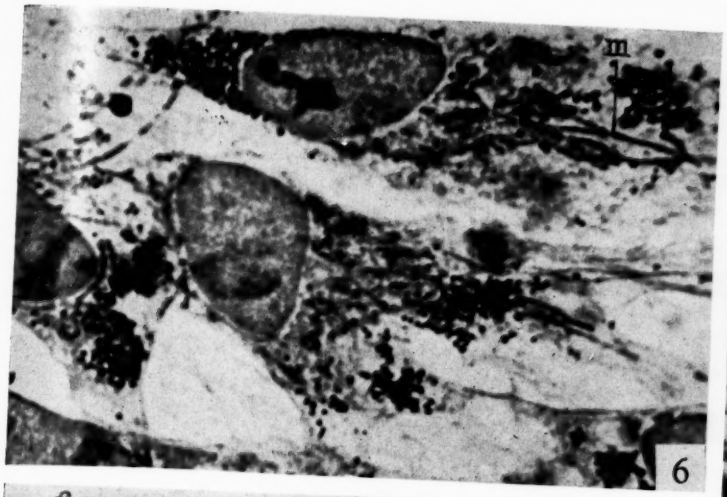
FIG. 8.—Fibroblasts after 2½ hours' exposure to saturated mustard gas vapor. They resemble those treated with the liquid agent except that the fat vacuoles, though enlarged, are much less distended. Mag. × 1,450.

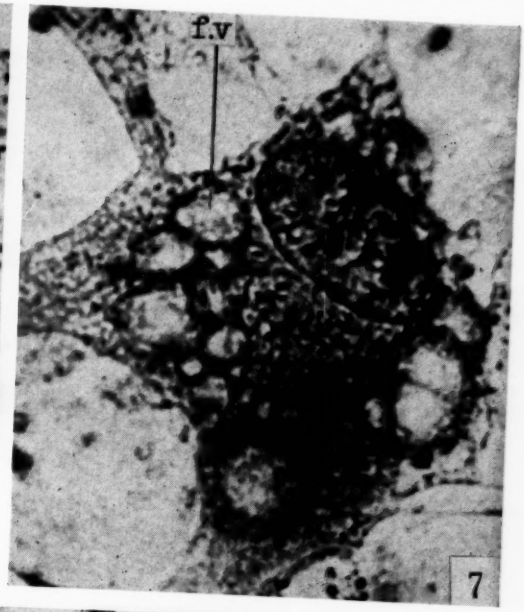
FIG. 9.—Fibroblasts after 2½ hours' exposure to mustard gas vapor in low concentration. The cells differ markedly from those subjected to the liquid or the saturated vapor in the great distortion of their nucleus and cytoplasm, the ground substance of which is relatively homogeneous. Note the swollen but deeply stained mitochondria. Mag.. × 1,450.

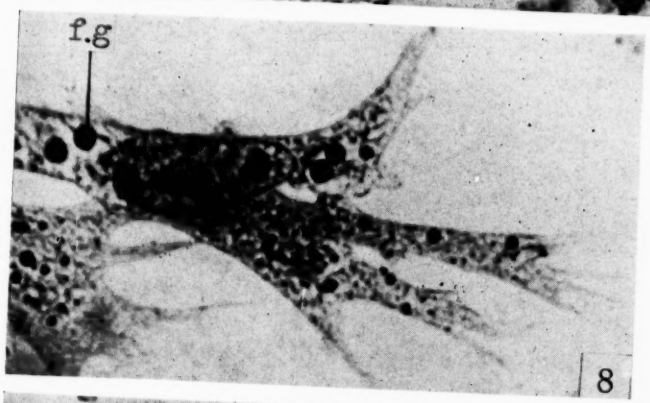
FIG. 10.—Fibroblast treated with pure thiodiglycol for 2½ hours. The cell is deformed, the nucleus shrunken and both nucleoplasm and cytoplasm have a flocculent appearance. Mag. × 1,450.

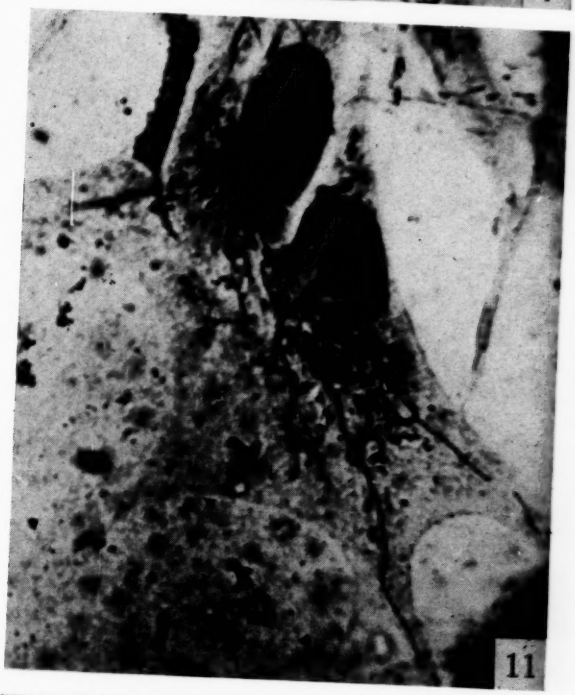
FIG. 11.—Cells treated with 0.15 per cent thiodiglycol for 2½ hours. They are almost unaffected (Fig. 6). Mag. × 1,450.

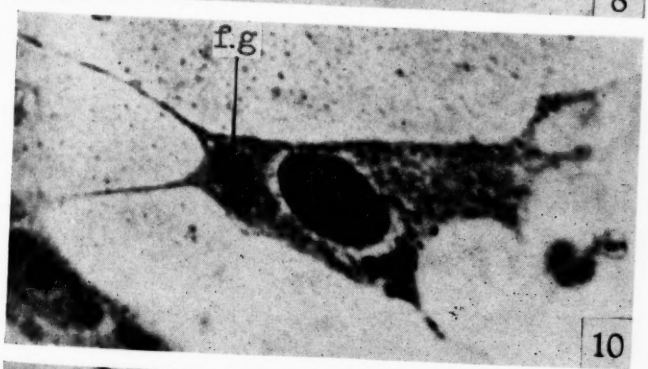
FIG. 12.—Cells treated with a mixture of dilute thiodiglycol and HCl. As compared with fibroblasts exposed to liquid mustard gas, the nuclei are somewhat shrunken and their outline is thicker, the reticular coagulum of the protoplasm is coarser, the fat globules are not distended and the remains of the mitochondria are more distinct. Mag. × 1,450.

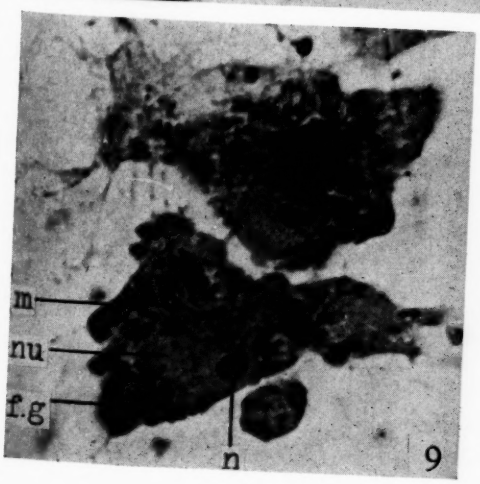


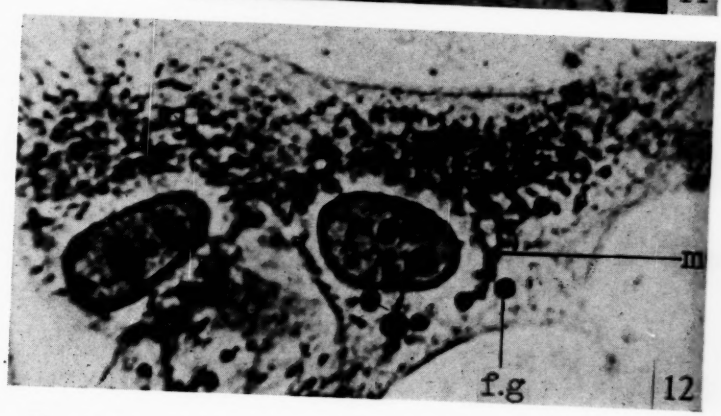




Figs. 6–12

iron hematoxylin (Fig. 8) the cells exposed to the vapor closely resemble those to which the liquid has been applied, except that the fat globules are much smaller and more intensely osmicated. Feulgen's method demonstrates that the nuclear structure and chromosomes are as well preserved as in cultures killed by contact with liquid mustard gas.

### III. The Effect of Mustard Gas Vapor in Low Concentration (6, 7)

To prepare cultures for these experiments, a small glass inlet tube, sealed at the outer end with paraffin wax, was inserted in the wax seal holding the coverslip to the hollow-ground slide. The tissue was allowed to grow for 2 days, then the wax blocking the outer end of the inlet tube was removed and with a fine pipette a small drop of liquid mustard gas was placed in the tube about half-way along its length; the outer aperture of the pipe was again sealed with paraffin wax and the culture placed either in the incubator or on the warm stage of the microscope for direct observation. Under these conditions, with the mustard gas contained in a capillary tube, considerable time elapses before equilibrium is reached, and the culture is thus exposed to a low concentration of vapor. Fifty-two cultures were treated.

These low concentrations of the vapor produce an entirely different cytological effect from that of the saturated vapor. The cells are not rapidly coagulated with little distortion of outline, but die slowly becoming contorted into diverse peculiar shapes.

A few minutes after the mustard gas has been placed in the inlet tube, the cells begin to withdraw their processes, sometimes leaving behind long, threadlike, branching strands of cytoplasm; meanwhile small blebs are protruded here and there, enlarge, change shape and are withdrawn into the cell body only to reappear elsewhere until the whole surface of the cell is slowly "bubbling." The cytoplasm, especially in the blebs, acquires a curious glassy look and has a greenish tinge with direct illumination. Fat globules in the blebs are in violent Brownian movement and the entire protoplasm seems to be softening.

About 20 minutes after the first application of the vapor, nearly all the cells have withdrawn their pseudopodia, are "bubbling" and have assumed fantastic forms. Many of the blebs break off and lie free in the medium and some of the cells round up completely and fall off the coverslip. After 1 to 3 hours according to the rate of diffusion of the gas from the capillary, "bubbling" ceases, vacuoles filled with fluid appear in the now motionless blebs and in the cell body, while the intravacuolar cytoplasm becomes very refractile and seems to coagulate; the nucleus is greatly shrunken and the nuclear membrane and nucleoli are very distinct. During the next 33 hours there is little further change.

The cultures give a positive reaction with $SeO_2$ which, however, is less intense than in cultures treated for the same time with saturated vapor.

In preparations fixed in osmic acid after contact with the vapor in low concentration, and stained with iron hematoxylin (Fig. 9), the ground substance of both nucleus and cytoplasm appears almost homogeneous as in the normal controls, thus contrasting strongly with the coarsely reticular protoplasm of cells treated with liquid mustard gas or the saturated vapor (Figs. 7 and 8). Until the final stages of degeneration the mitochondria appear surprisingly normal, though slightly swollen and rather closely aggregated in the interior of the deformed cell. Later they become very bloated and fragmentary and lose their staining power.

Drastic changes in the chromatin structure of the nucleus are revealed by the Feulgen technic (Fig. 15). In extreme cases the nuclei, which are very distorted, contain hardly any Feulgen-positive material, but usually the nuclear content appears as a scanty, coarse and very irregular red network. The mitotic figures are often nearly unrecognizable, so deformed are the chromosomes.

### IV. The Effect of the Hydrolysis Products (7)

The hydrolysis products of mustard gas were applied to the living cultures in contact preparations like those made with the liquid dichlordiethyl sulphide. In some experiments pure thiodiglycol was used (22 cultures); in others (16 cultures) the pure liquid was diluted with Pannett and Compton's saline to give a final concentration in the culture medium similar to that which would result from the hydrolysis of liquid mustard gas in contact with the culture; in a third set (21 cultures) hydrochloric acid was added to the same concentration of thiodiglycol as was used for set 2, in the proportion of 2 molecules of HCl to one of thiodiglycol; in a fourth set (21 cultures) the action of HCl alone was investigated, the concentration of the acid being the same as in set 3.

These concentrations of thiodiglycol and hydrochloric acid were computed on the assumptions that the solubility of mustard gas in water, and therefore in the culture medium, is 0.1 per cent at 37° C. and that all dissolved mustard gas is completely hydrolyzed under the conditions of the experiment. One liter of a saturated aqueous solution of mustard gas should thus contain 0.77 gm. of thiodigly-

col and 0.46 gm. of HCl. When a culture is placed in contact with such a solution of thiodiglycol and HCl, these substances must diffuse from the solution into the clotted culture medium; since the volume of the clot roughly equals that of the drop of fluid added, the experimental solutions were made in double strength, so that the final concentrations were thiodiglycol: 0.154 gm. (0.13 cc.) and HCl: 0.092 gm. (2.5 cc. concentrated acid) per 100 cc. of saline.

1. *Pure thiodiglycol.*—Although pure thiodiglycol is toxic, its effect is far less severe than that of liquid mustard gas. The immediate "fixation" of the protoplasm and its engorgement with refractile material which characterize the action of liquid mustard gas do not occur with pure thiodiglycol. Instead, after about 3 minutes' contact with the agent, the cells look glassy and greenish with direct illumination; after 2½ hours' incubation they are very distorted and sometimes disintegrate, and there is now a partial coagulation of the protoplasm.

The cells give an intense reaction with $SeO_2$ at this stage; large, dark granules, apparently formed in the fat globules, are seen and in addition, the entire cell is filled with a finely granular, yellowish-brown precipitate.

In cultures fixed in osmic acid after 2½ hours' contact with thiodiglycol, and stained with iron hematoxylin (Fig. 10), the nuclei are shrunken and deeply stained and the ground substance of both nucleus and cytoplasm has a flocculent appearance very different from that of either the normal or mustard gas-treated cells. The osmicated fat globules, many of which have fused together, lie near the periphery of the cell and sometimes even bulge from the surface. The nucleus, when stained by Feulgen's method, is filled with a coarse, dense red precipitate in which lie the colorless nucleoli; its chromatinic structure has little resemblance to that of cells exposed to liquid mustard gas. In the mitotic figures the chromosomes are swollen and clumped.

2. *Dilute thiodiglycol.*—Cells are almost unaffected by a 0.15 per cent solution of thiodiglycol even after 2½ hours' contact (Figs. 6 and 11). The only abnormality detected is a slight swelling of the mitochondria.

The cultures give a feeble reaction with $SeO_2$, and the cells in the zone of outgrowth contain a few small dark granules (Fig. 18, Plate III).

3. *Dilute thiodiglycol + dilute hydrochloric acid.*—The mixture of thiodiglycol and HCl immediately "fixes" the cells without appreciably distorting their shape, but produces a much coarser coagulation of the protoplasm than liquid mustard gas and does not infiltrate the cells with refractile droplets. The cultures react even less with $SeO_2$ than those treated with dilute thiodiglycol.

In cells fixed with osmic acid and stained with iron hematoxylin (Fig. 12), the nucleus, which is somewhat shrunken, is bounded by a very thick dark line, there is some fusion of the fat globules, and the mitochondria, which are often quite distinct, are abnormally short and thin. The Feulgen method shows that the chromatin granules and the chromosomes of mitotic cells are rather swollen.

4. *Dilute hydrochloric acid.*—The cytological effect of 0.1 per cent HCl is indistinguishable from that of the mixture of HCl and thiodiglycol. The treated cultures do not react with $SeO_2$.

## II. The Effect on Cell-Growth of Adding Small Concentrations of Mustard Gas to the Culture Medium.

### TECHNIC

Cultures of the choroid and sclerotic of 11 to 12 day chick embryos were made by the hanging drop method described in Part I. After 48 hours' growth in normal medium, the cultures were grouped into equivalent sets of six and transferred for various periods to a medium containing different concentrations of mustard gas. For histological examination the preparations were fixed in 5 per cent acetic alcohol for 3 minutes, stained with Ehr-

### DESCRIPTION OF FIGURES 13 TO 18

FIG. 13.—Prophase and vegetative nucleus in normal fibroblasts. Susa and Feulgen stains. Mag. × 1,450.

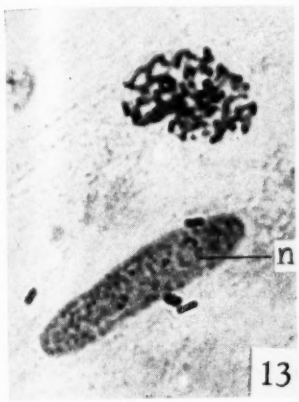
FIG. 14.—Prophase and vegetative nucleus in a culture exposed to liquid mustard gas for 2½ hours. Note the remarkably normal appearance of the chromatin structures (Fig. 13). Susa and Feulgen stains. Mag. × 1,450.

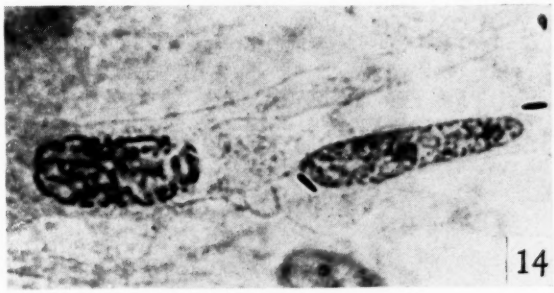
FIG. 15.—Fibroblast nuclei in a culture subjected for 2½ hours to mustard gas vapor in low concentration. Note the intense distortion of the entire nuclear structure. Susa and Feulgen stains. Mag. × 1,450.

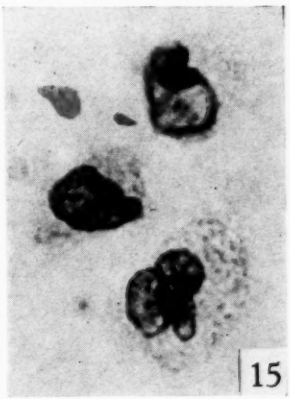
FIG. 16.—Cells in a culture treated with liquid mustard gas for 2½ hours and then with the $SeO_2$ solution. Note the coarse and abundant precipitate in the cells. $SeO_2$ and Ehrlich's hematoxylin stains. Mag. × 1,450.

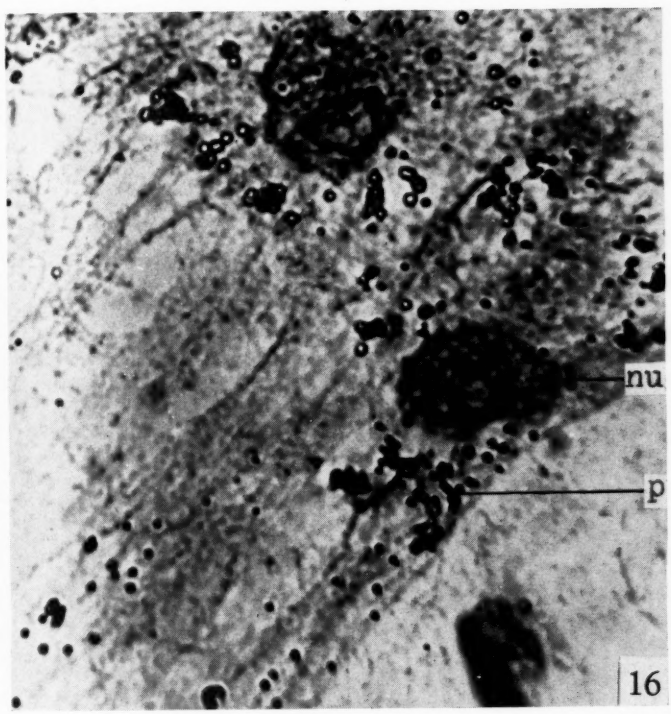
FIG. 17.—Cells treated with saturated mustard gas vapor for 2½ hours and then with $SeO_2$ solution. The precipitate is finer than after exposure to liquid mustard gas. $SeO_2$ and Ehrlich's hematoxylin stains. Mag. × 1,450.

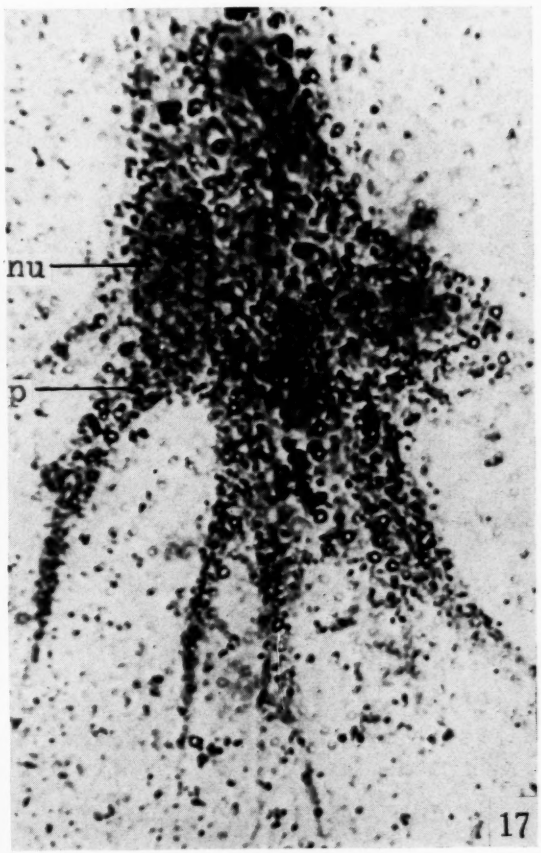
FIG. 18.—Cells treated with 0.15 per cent thiodiglycol for 2½ hours and then with $SeO_2$ solution. The precipitate cipitate has formed in the cells, but much less than in cultures treated with liquid mustard gas or the saturated vapor. $SeO_2$ and Ehrlich's hematoxylin stains. Mag. × 1,450.

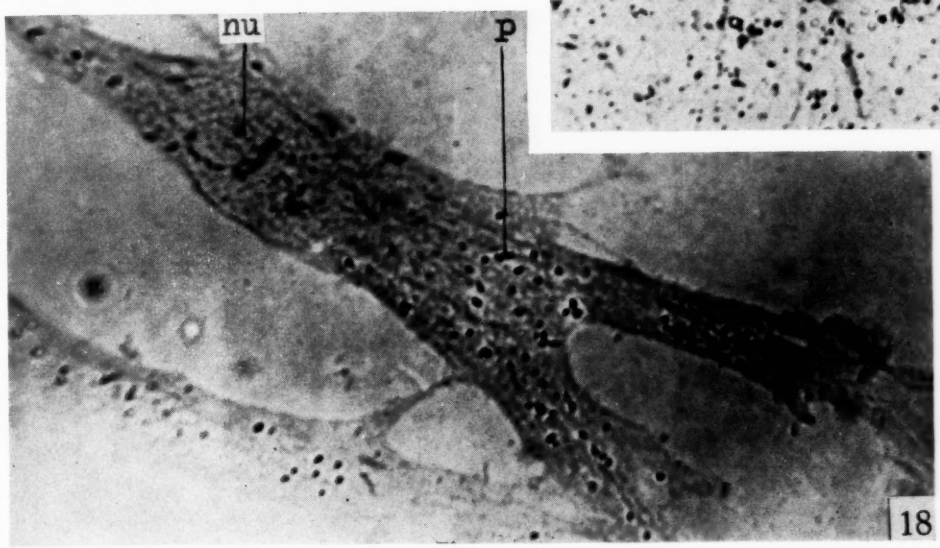



Figs. 13–18

lich's hematoxylin and mounted whole in Canada balsam.

## RESULTS

To find the minimal (immediate) lethal concentration of mustard gas for cells *in vitro,* 0.1 cc. of liquid mustard gas was stirred vigorously in a mechanical stirrer with 8.8 cc. of fowl plasma for 15 minutes at room temperature; this solution was diluted further with plasma in such a way that the addition of an equal volume of embryo extract would produce a clot containing the required concentration of mustard gas. A set of cultures were grown for 48 hours in each of the experimental media thus prepared and were then fixed and stained. Four experiments were made, the results of which are summarized in Table II.

TABLE II: SUMMARY OF EXPERIMENTS TO FIND (A) THE MINIMUM (IMMEDIATE) LETHAL DOSE OF MUSTARD GAS AND (B) WHETHER MUSTARD GAS IS DETOXICATED BY INCUBATION IN PLASMA. VERY GOOD GROWTH IS DENOTED BY +++; FAIR BUT SUBNORMAL GROWTH BY ++; POOR GROWTH BY +; NO GROWTH BY −.

| No. of set | Concentration of mustard gas in medium | No. of cultures treated | Results after 48 hours' cultivation |
|---|---|---|---|
| 1 | None (controls) | 24 | 1:+; 23:+++ |
| 2 | 0.5 γ/cc | 6 | +++ |
| 3 | 5 " | 12 | ++ |
| 4 | 50 " | 12 | 1:−; 11:++ |
| 5 | 100 " | 6 | ++ |
| 6 | 200 " | 18 | 5:−; 13:+ |
| 7 | 200 " * | 6 | ++ |
| 8 | 350 " | 6 | + |
| 9 | 350 " * | 6 | +++ |
| 10 | 500 " | 6 | − |
| 11 | 500 " * | 6 | ++ |
| 12 | 1000 " | 6 | − |
| 13 | 1000 " * | 6 | + |

*Incubated in plasma for 24 hours before use.

Very slight growth was obtained after 48 hours' cultivation in a concentration of mustard gas of 350 γ / cc. (set 8, Table II) and none in a clot containing 500 γ / cc. (set 10), indicating that the immediate minimal lethal concentration is between 350 and 500 γ / cc.

An interesting feature of cultures grown in sublethal concentrations is the many abnormal mitotic figures which they contain (10) (Figs. 19 to 24). These mitotic abnormalities are most conspicuous at the 50 and 100 γ / cc. levels (sets 4 and 5). At greater dilutions the abnormalities are usually less severe, though some fine multipolar divisions (Fig. 19) are found even in the lowest concentration used (0.5 γ / cc.). Above the 100 γ / cc. level the number of abnormalities declines because mitosis is largely suppressed. The most obvious mitotic anomalies (Figs. 19 to 24) are multipolar figures (Figs. 19, 24), chromosome fragmentation (Fig. 23), chromosome lag (Fig. 22) during anaphase, and a very eccentric position of the spindle in the cytoplasm (Fig. 20). Vegetative cells containing several nuclei varying greatly in size (Fig. 21) are common and all stages in their formation by abnormal mitosis may be seen.

When the solution of mustard gas in plasma is incubated for 24 hours before use, its toxicity is greatly reduced. The four sets of cultures (7, 9, 11 and 13, Table II), transplanted to medium containing such previously incubated material, all grew much better than the corresponding sets in medium to which the same concentrations of mustard gas had been added immediately before use. This was particularly striking in sets 11 and 13 which grew, though subnormally, in concentrations which were lethal when added to the plasma immediately before culturing (sets 10 and 12, Table II).

Experiments were made to study the effect on cell-survival of continuous cultivation in concentrations of mustard gas below that immediately fatal to the tissue. Owing apparently to a seasonal change in the fat content of the plasma, it was unsatisfactory to stir liquid mustard gas directly into the plasma as in previous experiments and it was therefore added in ethyl alcoholic solution in such a way that the final concentration of alcohol in the clot would not exceed 1 part in 2,000; this quantity of alcohol was added also to the control cultures. All the cultures were grown in normal medium for 48 hours and were then transferred to the experimental media for 1 to 5 passages. To test the viability of the cells treated with mustard gas when returned to a normal environment, three sets of cultures (7, 8 and 9, Table III) which had been grown in the mustard-containing clot for 2, 4 and 6 days respectively, were placed in normal medium for 2 days after which they were fixed and stained.

Table III shows that after 8 days' growth in a clot containing 50 γ / cc. mustard gas, *i.e.,* less than one-seventh of the immediately lethal concentration, 9 out of 12 cultures (sets 5 and 6) were dead and the rest growing very feebly, while all those in a concentration of 100 γ / cc. (set 4) were dead by the end of the 4th passage. The cultures which were restored to normal medium after 4 days in a clot containing 50 γ / cc. of mustard gas, grew almost normally, those transferred after 6 days showed slight improvement and those after 8 days no sign of recovery.

## DISCUSSION

The observations described above show that the cytological effects of liquid mustard gas or its saturated vapor are very different from those produced by low concentrations of the agent either as vapor or when dissolved in the culture medium.

Both liquid mustard gas and its saturated vapor rapidly kill the cells by coagulation or "fixation" with little distortion of their shape and with remarkably good preservation of the nuclear chromatin and of the chromosomes. This relatively good preservation of the chromatin structures, which also characterizes the central areas of mustard gas lesions in human and animal skin, could be achieved by a rapid and complete precipitation of the nucleoproteins such as that described by Berenblum (3). This fixation effect does not prevent the mustard gas from infiltrating the cells and accumulating in the fat vacuoles.

Unlike the pure liquid or the saturated vapor, dilute concentrations of mustard gas do not coagulate the protoplasm but seem to reduce its viscosity and render the chromatin structures almost unrecognizable. This type of cell damage might well

TABLE III: THE EFFECT ON CELL-SURVIVAL OF CONTINUED CULTIVATION IN CONCENTRATIONS OF MUSTARD GAS BELOW THE IMMEDIATELY LETHAL CONCENTRATIONS. THE OUT-GROWTH OF A FEW CELLS ONLY DENOTED BY ±; OTHER SYMBOLS AS IN TABLE II

| No. of set | Concentration of mustard gas in medium | No. of cultures treated | Results: End of 1st passage (2 days) | End of 2nd passage (4 days) | End of 3rd passage (6 days) | End of 4th passage (8 days) | End of 5th passage (10 days) |
|---|---|---|---|---|---|---|---|
| 1 | Control | 6 | +++ | +++ | +++ | +++ | +++ (fixed) |
| 2 | Control+ 1:2000 alcohol | 6 | +++ | ++ | ++ | ++ | ++ (fixed) |
| 3 | " | 12 | +++ (fixed 6) | +++ | +++ | +++ (fixed) | |
| 4 | 100 γ/cc+ 1:2000 alcohol | 12 | + (fixed 6) | 1:−;5:+ | 2:−;4:+ | − (fixed) | |
| 5 | 50 γ/cc+ 1:2000 alcohol | 12 | ++ (fixed 6) | + | 1:−;5:+ | 4:−;2:± (fixed) | |
| 6 | " | 6 | ++ | + | + | 5:−;1:+ | 5:−;1:± (fixed) |
| 7 | " | 6 | ++ | + (transferred to normal medium) | ++ (fixed) | | |
| 8 | " | 6 | ++ | + | + (transferred to normal medium) | + (fixed) | |
| 9 | " | 6 | ++ | + | + | 4:−;2:+ (transferred to normal medium) | 4:−;2:± (fixed) |

That the infiltrating material is mustard gas itself and not one of its products is indicated by its reaction with selenium dioxide. This reagent also gives a colored precipitate with thiodiglycol. The concentration of thiodiglycol to be expected in the tissue cultures would not be higher than 0.08 per cent however, and control experiments showed that when this and even higher concentrations of the hydrolysis product were applied to the cultures, the subsequent reaction with selenium dioxide was far less pronounced than in similar cultures treated with mustard gas. The reagent forms no precipitate with dichlordiethyl sulphone, the first oxidation product of mustard gas. It seems evident, therefore, that the coarse precipitate and deep coloration which selenium dioxide produces in cells treated with mustard gas indicate the presence of dichlordiethyl sulphide itself.

The results described in Part II show that fibroblast cultures die in concentrations of mustard gas above 0.035 per cent. It might have been expected that in such dilution the mustard gas would become completely hydrolyzed and thus harmless; as stated above, if plasma containing the same concentration is incubated for 24 hours before being be caused by some interference with the enzyme systems like that observed in biochemical studies by Peters (15) and by Dixon (4).

DESCRIPTION OF FIGURES 19 TO 24

All the cultures were fixed in 5 per cent acetic alcohol and stained with Ehrlich's hematoxlyin.

FIG. 19.—Tetrapolar anaphase in a culture grown for 48 hours in medium containing 5 γ / cc. mustard gas. Mag. × 1,700.

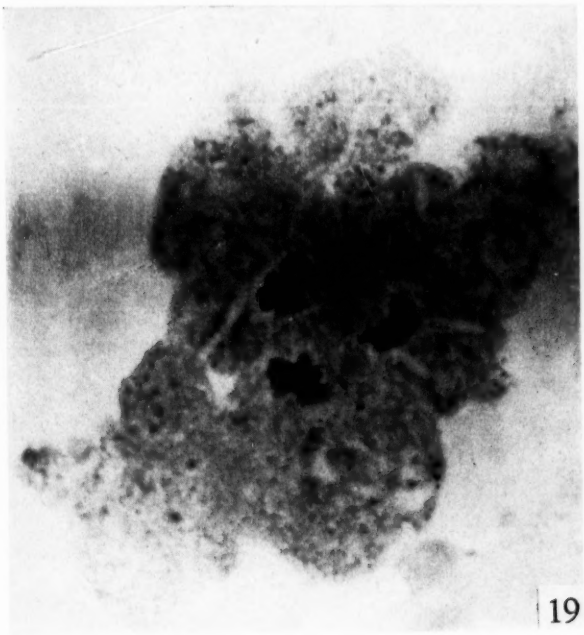
FIG. 20.—Metaphase in the same culture showing an eccentrically placed spindle. Mag. × 1,700.

FIG. 21.—Multinucleate cell in a culture grown for 48 hours in a medium containing 50 γ / cc. mustard gas. Note the variation in size of the nuclei. Mag. × 570.

FIG. 22.—Abnormal telophase from the same culture as that shown in Fig. 21. Note the lag in the separation of the daughter chromosomes. Mag. × 1,700.

FIG. 23.—Abnormal mitosis from the same culture showing fragmentation of the chromosomes. Mag. × 1,700.

FIG. 24.—Tripolar metaphase from a culture grown for 48 hours in medium containing 100 γ / cc. of mustard gas. Mag. × 1,700.



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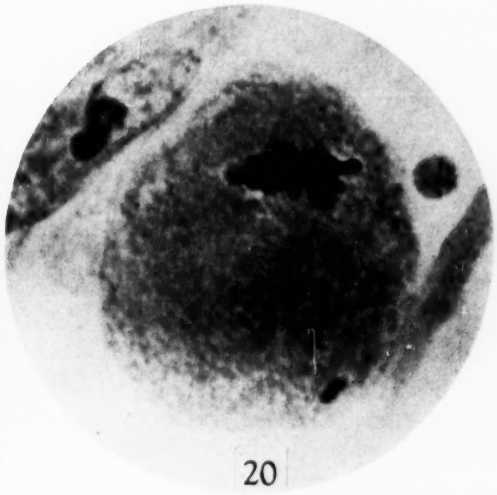

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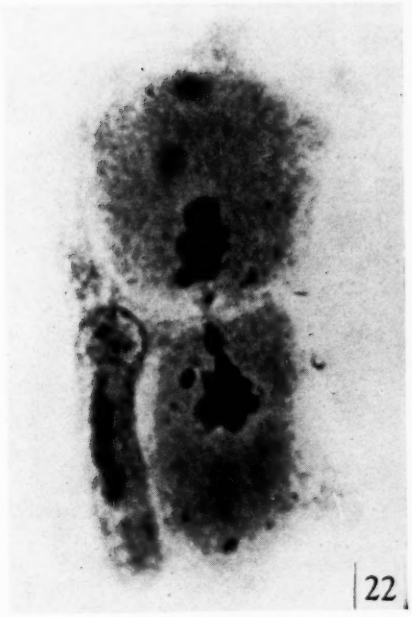

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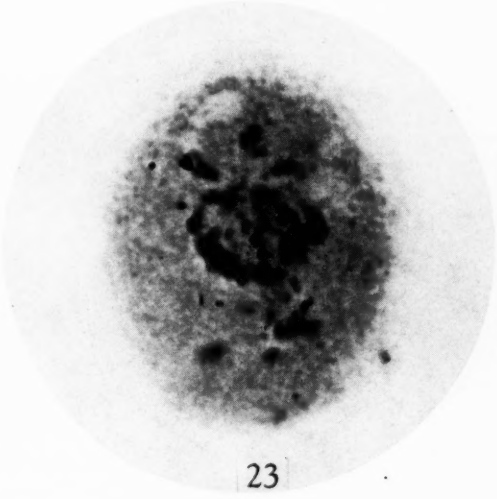

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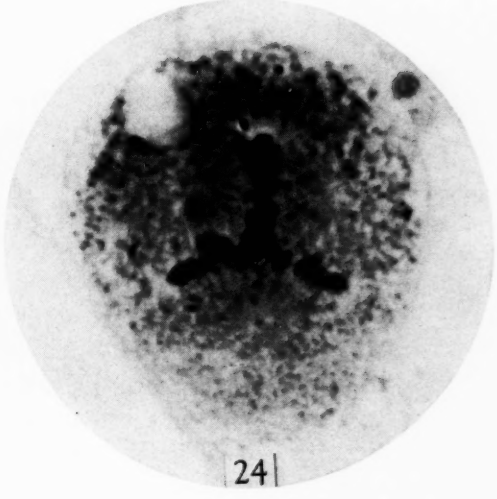

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Figs. 19–24

added to the cultures, the mustard gas is in fact largely detoxicated so that the cells not only survive but grow nearly as well as in the controls (Table II, set 9). If, however, treble the concentration of mustard gas is mixed with the plasma which is then incubated for 24 hours before use, the resulting medium retains considerable toxicity and greatly impairs cell growth (Table II, set 13). Observations on the interaction between mustard gas and plasma protein have been made by C. B. A. and will be published elsewhere.

The experiments on the delayed lethal effect of small concentrations of mustard gas (Table III) show that the agent has a cumulative action, possibly due to the storage of some toxic product in the cells. A similar cumulative effect is seen in mouse skin treated with repeated small doses of mustard gas (12). Although the poisoning process can be arrested in its earliest stages if the tissue is returned to normal medium, later all power of recovery is lost.

The many abnormal mitotic figures seen in cultures grown in medium containing sublethal concentrations of mustard gas, closely resemble those described by Strangeways (16) in tissue cultures irradiated with x-rays. They are also similar to those found in the skins of mice subjected to continuous irritation with dilute mustard gas (12). Severe mitotic disturbances resulting from mustard gas poisoning have also been described by Koller (14) in the pollen grains of *Tradescantia,* and the interesting genetical experiments of Auerbach and Robson (2) showed that mutations can be induced by exposing *Drosophila* to suitable concentrations of mustard gas vapor.

## SUMMARY

1. Warm-stage observations show that liquid mustard gas or its concentrated vapor immediately coagulates and kills living cells with little distortion of form. The liquid rapidly enters the fat vacuoles which become enormously extended, and finally infiltrates the entire protoplasm with droplets.

2. A histochemical technic based on the reaction of mustard gas with selenium dioxide makes it possible to identify the infiltrating substance as mustard gas itself and not one of its hydrolysis or oxidation products.

3. Liquid mustard gas readily accumulates in cells previously coagulated by heat or formalin, but if the fat is extracted from such coagulated cultures, contact with the liquid produces no engorgement.

4. Low concentrations of mustard gas vapor cause slow death of the cells with softening and disintegration of the cytoplasm, and enormous distortion of the cell outline, of the chromosomes in mitotic cells and of the chromatin structure of resting nuclei.

5. None of the hydrolysis products of mustard gas produces exactly the same cytological effects as the agent itself.

6. The minimal *immediate* lethal concentration of mustard gas for fibroblast cultures *in vitro* is between 0.035 and 0.005 per cent. At this concentration, it is partly detoxicated by incubation in excess plasma at 37° C. for 24 hours.

7. Cells *in vitro* are killed by continued cultivation in a concentration less than one-seventh of that which is immediately lethal. Degeneration can be arrested during the early stages of poisoning if the cultures are transferred to normal medium, but later all power of recovery is lost.

8. Cells grown in sub-lethal concentrations of mustard gas exhibit many abnormal mitotic figures.

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# Physiological Studies on Tumor-Inhibiting Agents

## III. Effect on Apparent Systolic Blood Pressure in Mice of the *Serrotia marcsecens* Tumor-Necrotizing Polysaccharide of Shear*†

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Patients treated with *Serratia marcescens* tumor-necrotizing polysaccharide show pronounced hypotension, lasting sometimes for days (3, 5, 6). It was of interest to determine whether a similar reaction occurs in tumor-bearing mice injected with this substance, especially since such mice exhibit reactions suggestive of circulatory disturbances, *i.e.*, prostration and depressed rectal temperature.

The procedure employed by Sobin (8) for measuring systolic blood pressure in the rat has been adapted by us to the mouse. Fig. 1 is a diagram of the apparatus we have employed. The tail-warmer plethysmograph and the occluding cuff were made of pyrex glass.[1] The inner (tail-containing) chamber of the plethysmograph had an inside diameter of 8 mm. and an overall length of 85 mm. Ureteral drainage tubing of 7.5 mm. diameter was tied into place in this chamber, care being taken that the rubber was neither too loose nor too taut. The left-hand opening was closed with a cork, leaving the right-hand opening free for insertion of the tail. The occluding cuff had openings 9 mm. in diameter. Drainage tubing of 10 mm. diameter was tied into place in this cuff. The length of tail occluded by the cuff was 15 mm. The capillary-indicating tube used to read changes in the volume of the tail had an inside diameter of 0.3 mm.

A smaller diameter-indicating tube is needed for the mouse than for the rat, and changes in the level of the meniscus are so small as to require that a small magnifying glass be placed in front of the meniscus. The indicator fluid used by Sobin was unsatisfactory in the smaller indicating tube used by us. One per cent potassium dichromate, plus 0.015 per cent aerosol to reduce surface tension, proved satisfactory. It was of course necessary to make sure that no air bubbles were present anywhere between stopcock 1 and the top of the column of dichromate in the capillary indicating tube.

The middle part of the mouse holder consisted of a cylinder, open at both ends. This cylinder was made of lumerith, a transparent cellulose acetate product sold in sheet form, which can be easily bent and cut. Any two surfaces can be molded together by placing a small drop of acetone between them and holding them firmly together for a moment. Lumerith sheets of 0.2 mm. thickness were used, into which were punched many small holes, so that the mouse would not become overheated. Since mice can easily chew their way through lumerith, the head end of the holder was made of a metal cap, long enough to contain the entire head of the mouse. A 0.5 cm. hole was drilled in the end of the cap to provide an air supply for the mouse. The metal cap and the middle of the holder were bound firmly together with cellophane tape. The tail end of the holder consisted of a cap made of lumerith, in the end of which a hole about 1 cm. in diameter was cut, through which the tail could project. The tail end of the holder was so made that it would slip easily but snugly over the open end of the middle part of the holder. It is usually an easy matter to induce a mouse to crawl into the opening in the middle part of the holder. With the mouse in place, the middle and tail parts of the holder were sealed together with cellophane tape.

With the mouse in the holder the tail was threaded through the occlusion cuff and into the space within the rubber membrane in the inner chamber of the plethysmograph. Threading of the tail into the plethysmograph was facilitated by closing stopcock 2, opening stopcock 1, pulling on the syringe and then closing stopcock 1. This opened up a wide passageway into the tail chamber. Stopcock 1 was then opened and the syringe pushed down somewhat to bring the rubber into contact with the tail.

* This research was supported by a grant from the Donner Foundation, Cancer Research Division.

† In the first paper in this series (Temperature Changes Produced in Mice with S. Marcescens Polysaccharide—Approaches to Tumor Chemotherapy, A. A. A. S., Washington, D. C., 1947, page 265), please read μg. (*micrograms*) wherever the term mg. appears.

[1] The tail-warmer plethysmograph and the occluding cuff were prepared by Mr. Thomas Walton, 4583 G. St., Philadelphia 20, Pa.

The tail was warmed at 42° to 43° C. for at least 10 minutes, usually 15 to 20 minutes. To take a reading the mercury was pumped up to 200 mm., stopcock 3 being closed. With stopcock 2 closed, pressure was placed on the syringe to push dichromate solution into the space between the glass and the rubber tubing and thus force a small amount of blood out of the tail. Immediately stopcock 3 was opened, occluding the tail at a time when it contained a minimum of blood. Stopcocks 1 and 2 were opened and the syringe moved until the dichromate meniscus in the capillary tube occupied an intermediate position. Stopcock 1 was then closed again, and the mercury in the sphygsomanometer allowed to fall slowly. The systolic pressure was read as that pressure where the meniscus showed a steady permanent rise in the capillary tube. With practice it was usually possible to distinguish this change from changes in the meniscus due to struggling of the mouse. In cases of doubt, readings were repeated. After two or three successive readings had been obtained on a given mouse the mouse was removed and released from the holder. When two successive readings were within 5 mm. of each other they were averaged. When they were further apart than 5 mm., a third reading was taken, and the systolic blood pressure was taken as equal to the average of the two highest values obtained. If no two readings within 10 mm. of each other were obtained, additional readings were made or the data were discarded.

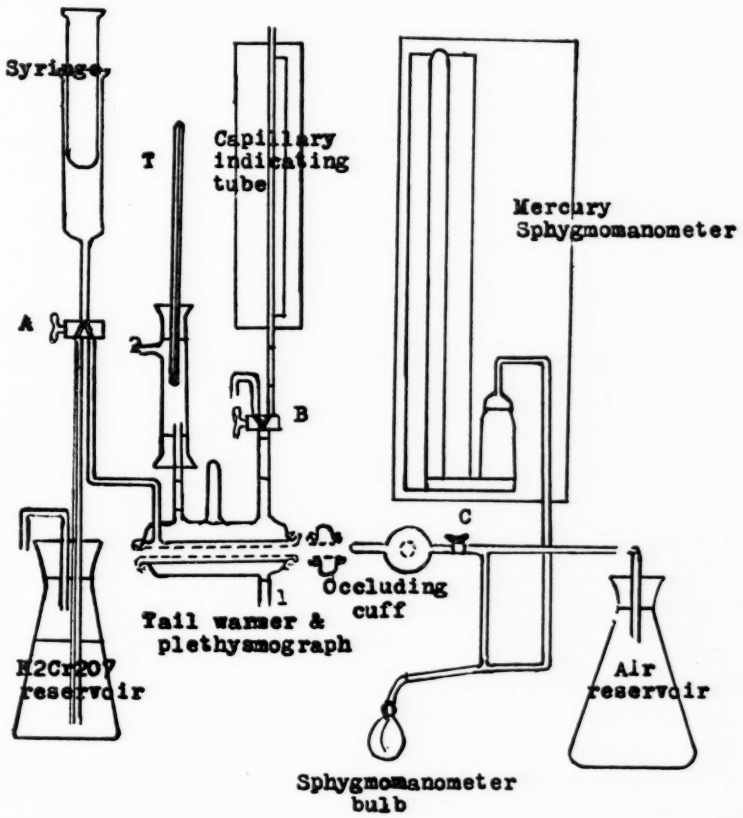


FIG. 1.—Apparatus employed in securing apparent systolic presure readings.
A and B = Two way glass stopcocks.
C = One way metal stopcock.
1 = Point of entrance of hot water into heating jacket of tail-warmer plethysmograph.
2 = Point of exit of hot water from chamber for indicating temperature of plethysmograph.
T = Centigrade thermometer.

The readings so obtained have not been compared with those obtainable on cannulation of a large artery. We have been primarily interested in determining whether *changes* in blood pressure occur in tumor-bearing mice injected with polysaccharide. Even for the purpose of judging whether changes in apparent systolic blood pressure had occurred the method was found to have definite limitations. The making of the systolic pressure determinations was found to produce definite injury to the tail, so that ordinarily it was not possible to secure more than three to six sets of readings on a given mouse. This injury was manifested: (a)

by decreased extent of movement of the dichromate meniscus and increased difficulty in securing readings several hours after the initial reading, even in mice not treated with polysaccharide and not showing any appreciable change in the apparent systolic blood pressure. These effects were noted on mice in which attempts were made to secure readings at hourly to half-hourly intervals, but were infrequent in mice used to obtain readings at 2 hour intervals, (b) by frequent failure to secure readings on the subsequent day in mice that were quite lively and showed normal rectal temperatures (c) by reddening of the tail and occasional rubbing off of part of the skin (d) by definite necrosis and falling off of part of the tail, occurring within the next day or so after the initial readings. Reactions such as those described above have been reported by Sobin (8) for young rats only.

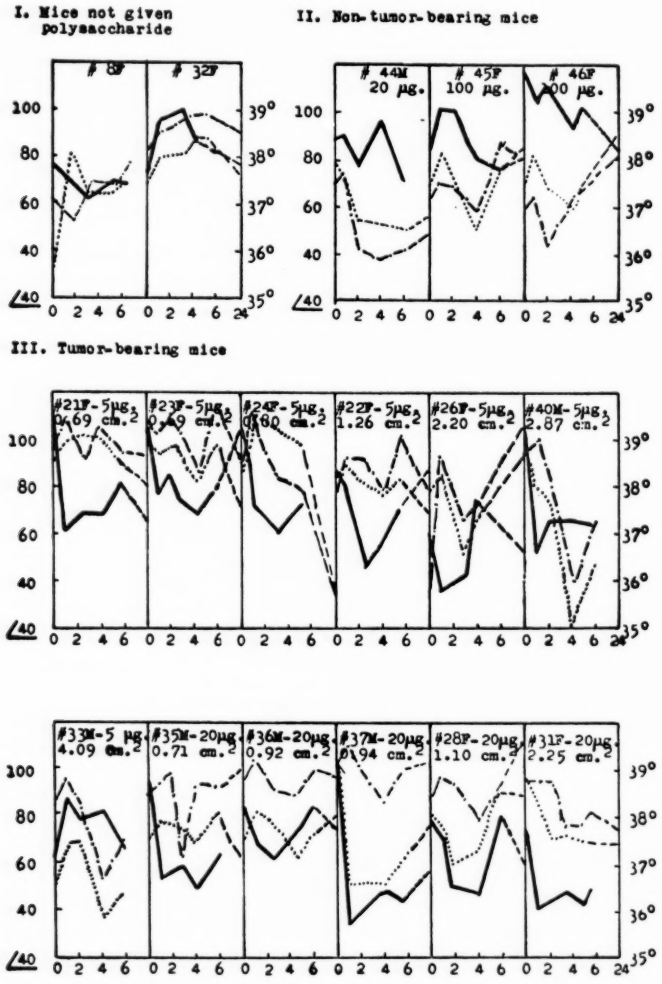


FIG. 2.—Changes in apparent systolic pressure and in rectal temperature in mice following intraperitoneal injection of *Serratia marcescens* polysaccharide preparation $P_3$.

Left-hand abscissæ and solid lines = Systolic pressure in mm. Hg.

Right-hand abscissæ = Rectal temperatures in degrees centigrade.

Dotted lines = rectal temperatures after S.P. determinations.

Dot-dash lines = Rectal temperatures after S.P. determinations.

Dash lines = To connect 6 hour readings with 24 hour readings (0 hours for one mouse equals 24 hours for preceding mouse).

Ordinates = Time in hours after injection of polysaccharide. Zero (0) times refer to readings obtained before polysaccharide injection.

Injection of polsyaccharide was followed by definitely lower systolic blood pressure readings. The lower these readings, the smaller was the movement of the dichromate meniscus. This was a limiting factor which prevented our securing a reading when the apparent systolic blood pressure was below 40 mm. Hg. Where we felt that injury of the tail had not been sufficient by itself to prevent the obtaining

of a reading we have designated failure to obtain a reading by the symbol /40 to indicate an apparent systolic pressure of less than 40 mm. Hg.

In the mouse, injection of polysaccharide is followed not only by fall in apparent systolic pressure but also by decrease in rectal temperature (2). The tail warming method of determining systolic blood pressure depends on the securing of maximum dilatation of tail arterioles as a result of warming the tail to 42° to 43° C. It is questionable whether maximum dilatation occurs in mice having definitely subnormal rectal temperatures (see Discussion). It should be noted here that following polysaccharide injection the fall in apparent systolic pressure in the mouse occurs some time before any appreciable change in rectal temperature has occurred, so that early systolic blood pressure readings by the tail method are probably closer to the systolic blood pressure in the large arteries near the heart than is the case for readings obtained later, when the rectal temperature is definitely depressed. Using thermocouples, we have made rectal temperature readings before and after each set of systolic blood pressure readings.

Tables II and III summarize data obtained following injection of 20 micrograms per mouse of polysaccharide (preparation $P3)_3$. Only three sets of systolic pressure readings were made on a given mouse, since it was thought this should decrease the likelihood of securing readings that were grossly erroneous because of injury to the tail. When a mouse died on the experimental day the tumor was removed and weighed that day. Otherwise the tumor was secured and weighed on the following day.

TABLE I: CHANGES IN APPARENT SYSTOLIC BLOOD PRESSURE AND IN RECTAL TEMPERATURE IN MICE FOLLOWING INTRAPERITONEAL INJECTION OF *Serratia marcescens* POLYSACCHARIDE PREPARATION P[3]

| External Area of Tumors: (Mouse Sarcoma 37) | | Zero (no tumor) | | Less than 0.5 sq. cm. | More than 0.5 sq. cm. | |
|---|---|---|---|---|---|---|
| Dose of polysaccharide: (gm. per mouse) | | 20 | 100 | 5 | 5 | 20 |
| No. of mice in group: | | 3 | 2 | 9 | 8 | 6 |
| Mean S.P.* before polysaccharide injection (mm. Hg.) | | 89 | 100 | 91.7 ±5.3 | 90.9 ±6.8 | 95.3 ±7.3 |
| Changes in S P. during 1st 6 hrs. after polysaccharide injection: (in mm. Hg.) | Av. for all readings all mice in group: | − 4.7 | − 3.5 | −14.2 ± 4.1 | −25.9 ± 4.7 | −40.2 ± 9.5 |
| | Av. for lowest S.P. value for each mouse: | −14.3 | − 15.0 | −29.2 ± 4.1 | −41.0 ± 2.3 | −50.3 ±11.0 |
| Av. of the times for each mouse at which the lowest S.P. reading was secured: (in hours) | | | | 3.4 ± 0.40 | 2.6 ± 0.71 | 3.1 ± 0.79 |
| Changes in rectal temp. during 1st 6 hrs. after polys. inj. (in C.) | Av. for all readings on all mice in group: | − 0.53 | 0.11 | − 0.32 ± 0.38 | − 0.05 ± 0.33 | − 0.68 ± 0.36 |
| | Av. for lowest reading for each mouse: | 0.73 | − 0.53 | − 0.85 ± 0.11 | − 0.74 ± 0.48 | − 1.40 ± 0.39 |
| Av. of the times for each mouse at which the lowest rectal temperature reading was obtained (hours): | | | | 3.50 ± 0.38 | 4.36 ± 0.32 | 4.50 ± 0.40 |

*For all tumor-bearing mice given polysaccharide*†

Av. time to reach lowest S.P. reading −2.91 ±0.36 hours

Av. time to reach lowest rectal temp. −3.81 ±0.24 hours

S. P. = Systolic pressure.

## EXPERIMENTAL FINDINGS

Figure 2 shows typical systolic pressure and rectal temperature readings obtained on Carworth Farms white mice treated with polysaccharide (preparation $P3)_3$. These data, and data obtained on additional mice, are summarized in Table I. Tumors (Sarcoma 37) of cross-sectional area less than 0.5 sq. cm. weighed less than 0.1 gm; those with cross sectional areas greater than 0.5 sq. cm. had weights ranging up to 2.5 gm. Plus, minus figures in the Tables represent standard errors.

From Fig. 2 and Tables I and II the following conclusions may be drawn:

A. Intraperitoneal injection of *Serratia marcescens* polysaccharide into tumor-bearing mice in amounts sufficient to cause considerable hemorrhage and necrosis in the tumor is followed within an hour or so by a definite to profound decrease in apparent systolic pressure.

B. Mice injected with polysaccharide usually show a decreased rectal temperature. This decrease generally occurs after the fall in systolic pressure, but was not always noted, even when a fall in systolic pressure had occurred.

C. The greater the amount of tumor tissue in a mouse the more profound and long-lasting was the decrease in both the apparent systolic pressure and the rectal temperature.

[3]We are indebted to the Chemotherapy Section, National Cancer Institute, Bethesda 14, Md., for samples of *Serratia marcescens* polysaccharide preparations P3 and P8.

D. The greater the amount of tumor tissue in a mouse the greater was the amount of prostration and diarrhea exhibited, and the greater was the likelihood that death would occur subsequent to administration of polysaccharide. This confirms Shear (7).

## DISCUSSION

When the data on rectal temperatures shown in Table II were analyzed to secure Table III some interesting facts were brought to light. In non-tumor-bearing mice, and in untreated mice with tumors weighing less than two grams the rectal

TABLE II: EFFECTS OF 20 MICROGRAMS PER MOUSE OF *Serratia marcescens* POLYSACCHARIDE PREPARATION $P_8$ ON APPARENT SYSTOLIC BLOOD PRESSURE AND RECTAL TEMPERATURE IN MICE BEARING TUMORS (SARCOMA 37) OF VARYING SIZES

| Mouse No. and sex | Wt. of tumor (gm. | S.P. readings (mm. Hg.) Control | 2 hr. value | 4 hr. value | Rectal temperature readings (°C.) Control values B | A | 2 hour values B | A | 4 hour values B | A |
|---|---|---|---|---|---|---|---|---|---|---|
| 112 F | Zero | 123 | 76 | 96 | 37.8 | 38.4 | 37.1 | 37.5 | 38.5 | 38.5 |
| 116 F | " | 70 | 82 | 50 | 36.9 | 39.2 | 38.1 | 39.5 | 38.2 | 38.9 |
| 117 F | " | 90 | 90 | 88 | 37.4 | 38.4 | 36.0 | 38.0 | 37.6 | 38.5 |
| 118 F | " | 104 | 75 | 65 | 37.9 | 38.6 | 36.6 | 39.4 | 38.1 | 38.1 |
| 233 F | " | 100 | 70 | 90 | 38.0 | 38.8 | 36.0 | — | 37.7 | — |
| 83 F | 0.04 | 95 | 83 | 81 | 39.0 | 38.4 | 36 8 | 37.9 | 38.1 | 36.8 |
| 79 F | 0.12 | 103 | 62 | 63 | 37.7 | 38.6 | 37.5 | 38.5 | 38.3 | 37.2 |
| 100 F | 0.16 | 103 | 91 | 83 | 37.1 | 37.4 | 36.0 | — | 35.6 | 34.4 |
| 80 F | 0.17 | 134 | */40* | 78 | 37.7 | 38.0 | 35.7 | — | 34.0 | 33.9 |
| 77 F | 0.30 | 106 | 61 | 80 | 37.6 | 38.8 | 38.0 | 37.8 | 37.0 | 37.7 |
| 106 F | 0.30 | 97 | 56 | 70 | 37.8 | 38 9 | 37.0 | 37.3 | 38.5 | 38.0 |
| 76 F | 0.38 | 80 | 42 | */40* | 38.7 | 39.3 | 37.9 | 38.0 | 35.0 | 38.1 |
| 130 M | 0 39 | 100 | 73 | */40** | 39.1 | 39.2 | 37.1 | — | 34.6 | Dead |
| 177 F | 0.60 | 80 | 66 | 73 | 37.5 | 39 3 | 38.0 | 37.6 | 33.8 | 33.4 |
| 75 F | 0.69 | 103 | 76 | 46 | 38.1 | 39.3 | 38 3 | 37.3 | 37.5 | 37.3 |
| 169 F | 1.05 | 80 | 85 | 60 | 35.7 | 37.6 | 34.8 | 35.3 | 34.9 | 35.1 |
| 247 F | 1.05 | 120 | 40 | 50 | 35.6 | 37.9 | 35.4 | 36.6 | — | 35.9 |
| 248 M | 1.10 | 66 | 63 | */40*† | 38.0 | 39.0 | 38.2 | 39.3 | 34.5 | 34.4† |
| 173 F | 1.35 | 111 | 51 | 42 | 38.9 | 38.5 | 37.3 | 37.7 | 33.4 | 33.5 |
| 176 F | 1.40 | 48 | 60 | 59† | 37.0 | 38.7 | 38.4 | 39.2 | 34.5 | 35.1† |
| 174 F | 1 73 | 89 | 90 | 55 | 38.1 | 39.3 | 38.3 | 37.3 | 37.5 | 37.3 |
| 245 F | 2.50 | 82 | 67 | 50 | 38.6 | 38.5 | 38.1 | 37.6 | 37.2 | 37.3 |
| 121 M | 2.51 | 72 | */40* | */40* | 37.9 | 37.9 | 37.1 | 37.6 | 35.3 | 33.8 |
| 105 F | 2.59 | 121 | 52 | 45* | 38.0 | 39.0 | 36.3 | 35.6 | 33.9 | 35.1* |
| 181 F | 2.70 | 80 | 52 | */40* | 38.3 | 38.7 | 36.6 | 35.7 | 36.4 | 35.5 |
| 107 F | 2.78 | 110 | 80 | Dead | 36.7 | — | 34.8 | — | Dead | |
| 201 M | 3.50 | 72 | 75 | */40* | 36.9 | 37.3 | 37.9 | 37.1 | 34.0 | 34.4 |
| 140 F | 3.53 | 56 | 62 | */40*† | 38.2 | 37.4 | 38.1 | — | 32.1 | 32.1† |
| 228 F | 3.66 | 50 | */40* | */40** | 36.1 | 35.7 | 35.9 | 36.3 | 32.6 | 32.1† |
| 250 F | 3.80 | 80 | 90 | */40*† | 37.7 | 37.5 | 36.4 | 36.1 | 35.7 | —† |

* Mouse died soon after reading was made
† Mouse found dead next morning
*/40:* No reading obtained—apparent systolic blood pressure less than 40 mm. Hg.
B: rectal temperature readings obtained *before* corresponding systolic pressure readings.
A: rectal temperature readings obtained *after* corresponding systolic pressure readings

E. As compared with tumor-bearing mice, normal mice showed much smaller changes in apparent systolic pressure and rectal temperature following injection of polysaccharide, even when considerably bigger doses were given. The dose of polysaccharide (preparation P8) required to kill over half of normal Carworth Farm mice is of the order of 500 micrograms per mouse, as compared with a dose of 20 micrograms for mice bearing tumors weighing more than 2 grams.

F. Mice with large tumors (1 gram or more) were more likely to show low original systolic pressure readings and low rectal temperature readings than either normal mice or mice with smaller tumors.

temperatures obtained after taking the systolic pressure readings were on the average nearly 1°C. higher than the corresponding rectal temperatures which had been obtained on these same mice prior to the tail heating which preceded the taking of the systolic pressure readings. This increase in rectal temperature is to be expected if an appreciable amount of heat is carried from the heated tail to the body of the mouse by the blood flowing through the tail. Conversely, the usual failure to secure such a rise in rectal temperature in mice with tumors of weight more than two grams indicates a sluggish blood flow through the tail in these mice.

Generally, an appreciable rise in rectal temperature, in connection with the tail heating, did not

occur in tumor-bearing mice at 2 and 4 hours after administration of polysaccharide, or in non-tumor-bearing mice at 4 hours after administration of polysaccharide. This indicates that a sluggish flow of blood in the tail is induced by the polysaccharide, particularly in tumor-bearing mice. The only other way in which this effect could be produced would be by a marked increase in rate of heat loss in the polysaccharide treated mice. This is very unlikely, since in mice administration of polysaccharide results in depression of both rectal temperature (2) and skin temperature (unpublished). Furthermore, Algire (1) has found that a sluggish blood flow and a marked decrease in functional vascular level occur in both sarcoma 37 and skeletal muscle following administration of the polysaccharide to mice prepared with a transparent chamber for microscopic observation.

Table III: Values Obtained When Rectal Temperature Readings Taken Just Before Systolic Pressure Readings are Subtracted from Corresponding Rectal Temperature Readings Obtained Just After These Same Systolic Pressure Readings

| Group of mice | No. of mice in group | Control values | 2 hr. values | 4 hr. values |
|---|---|---|---|---|
| Normal mice | 4 | 0.90 | 1.18 | 0.40 |
| Mice with tumors of wt. 0.04–1.73 gm. | 12 | $0.97 \pm 0.23$ | $0.23 \pm 0.22$ | $0\ 09 \pm 0.20$ |
| Mice with tumors of wt. 2.50–3.80 gm. | 7 | $0.06 \pm 0.20$ | $0.19 \pm 0.24$ | $0.17 \pm 0.34$ |

Decrease in ability of the tail to convey heat to the body of the mouse, cold skin, lowered rectal temperature, and the observations of Algire all indicate that appreciable peripheral vasoconstriction occurs following administration of polysaccharide. The heated tail method of securing systolic pressure readings depends for its reliability on the securing of complete arterial and arteriolar relaxation in the tail as a result of heating the tail to 42° to 43° C. It would appear unlikely that such relaxation occurs in polysaccharide treated mice; the readings in such mice may be expected to be appreciably lower than they would have been if complete arteriolar relaxation in the tail had been secured. Nevertheless we feel that the evidence is in favor of a generalized fall in blood pressure being produced in mice by polysaccharide treatment, since in most of the readings taken within 2 hours after polysaccharide administration, in which an apparent lowering of systolic blood pressure occurred, there was an accompanying increase in rectal temperature, subsequent to tail-heating of at least 0.5° C. (*See* Nos. 40, 33, 35, 36, 37, 28 and 31 of Fig. 1).

The definite correlation of the apparent systolic blood pressure readings with: (a) the degree of prostration and diarrhea exhibited by these mice; (b) the rectal temperature readings; and (c) the likelihood of death occurring subsequent to injection of polysaccharide indicate that apparent systolic blood pressure readings as obtained by the heated-tail method may be used as an objective criterion of the physiological condition of the mouse, even though they are probably of little value in judging the absolute level of systolic pressure in large arteries near the heart in tumor-bearing, polysaccharide-treated mice.

The injury to the tail of the mouse produced by the heated-tail method of securing systolic pressure readings is a matter of some interest, especially since it is apparently much less likely to occur in the rat. The mouse has a very high metabolic rate in relation to *body weight* (4). Heating of the tail to 42° to 43° C. may be expected to raise the metabolic rate in the tail still further. It seems to us likely that the tail injury produced by the procedures employed in taking systolic pressure readings is due to severe anoxia. If we take Herrington's values (4) for the rat, and convert these into values *per kgm. of body weight* we find that the metabolic rate for a 200 gm. rat is only about 40 per cent of that of a 20 gm. mouse, and the metabolic rate of a 100 gm. rat is about 50 per cent of that of a 20 gm. mouse. From this we would expect that the procedures involved in securing systolic pressure readings in a heated-tail would be more likely to produce irreversible damage by anoxia in the mouse than in the rat.

## SUMMARY

1. A method is described for securing systolic blood pressure readings in the heated tail of the non-anaesthetized mouse.

2. These readings were depressed in Carworth Farms white mice injected with *Serratia marcescens* tumor-necrotizing polysaccharide.

3. The extent of the depression was correlated with the amount of tumor tissue (Sarcoma 37) in the mouse, and with the dose of polysaccharide given.

4. The question as to whether the depression in apparent systolic pressure was due primarily to a generalized fall in blood pressure or to vasoconstriction in the tail is discussed.

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# The Biochemistry of Benzpyrene

## III. The Quantitative Estimation of the Metabolites.*

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### INTRODUCTION

The first two papers of this series gave evidence for the chemical changes undergone by 3,4-benzpyrene in the tissues of the mouse. A number of metabolites were described and provisionally named, $X_1$, $X_2$, $F_1$, and $F_2$. Of these, the chemical nature is known only of $F_2$, this being, in the case of mice, 8-hydroxybenzpyrene. Furthermore, it was shown that the X-derivatives are the first formed. It has since been shown that these are formed at the site of application of the benzpyrene (1).

In order to further the studies of these phenomena, an improved technic for the separation of the metabolites has been evolved and a system for their quantitative estimation developed.

### The Separation of the Metabolites

Throughout the method to be described allowance must be made for the relative instability of the metabolites and the fact that normal tissue components are liable to interfere in the process. Previously, a method for separation by partition chromatography has been described (2). As, however, the removal of the adsorbed material from the adsorbate cannot be carried out satisfactorily, this method has been discarded, and attention concentrated on the separation of the metabolites by way of their different solubilities in varying solvents. This method, with the improvements noted below has been found satisfactory.

Ultimately all preparations are destined for examination by ultraviolet light. Therefore, the reagents used must be checked for fluorescence before use. In actual practice, it has been found that even the best commercial reagents are rarely free from fluorescence due to impurities, and purification methods have had to be developed.

*Extraction of tissues.*—In the case of painted skins, it has been found advantageous to remove any loosely adherent benzpyrene by preliminary washings with benzene. Thereafter, the technic is the same for all tissues. After mincing very finely, small quantities not exceeding 1 gm. being used, the tissues are repeatedly extracted with 7 to 10 cc. lots of 70 per cent acetone (70 cc. acetone, 30 cc. glass-distilled water). The 70 per cent acetone is found to give a better extraction than pure acetone. Extractions are continued until both acetone and the tissue fail to fluoresce under the ultraviolet lamp.

The acetone extracts are pooled and evaporated under reduced pressure in the vessels described previously (3). To prevent loss from frothing during this process, 0.5 to 1.0 cc. of petrol ether (b.p. above 120° C.) is added. Thereafter the procedure is that described in Fig. 1.

The only point needing special mention is the fact that $F_1$ and $F_2$ still require to be separated from benzpyrene by chromatographic methods, since, unlike the X metabolites, no suitable solvents have been found to separate them. The choice of method followed must be determined by the experiment in hand.

Using these methods, solutions of the metabolites are obtained, and after clearing by centrifugation are ready for quantitative estimation.

### The Optical Estimation of the Metabolites

Although the following methods are based on the quantitative extinction spectra of purified substances, the amounts recovered from mouse tissues are rarely sufficient to allow the production of absorption spectrograms which can be directly estimated by photometric methods. Therefore, the indirect method of spectro-fluorometry has been used. Such a method has its limitations, but if these are borne in mind, the results are generally of the same order of precision as those in biological experiments.

No special experimental method can be recommended for the fluorescence spectrophotometry, as it depends upon the laboratory facilities available. Whatever method is used must be the subject of a period of trial and error until reliable results can be consistently obtained.

The problem under study is concerned with 5

* Note: Because of accelerated production schedule, proof of Dr. Weigert's paper has not been read in his laboratory.

† The Editors of *Cancer Research* announce with sincere regret that Dr. Weigert died April 13, 1947. The present paper was prepared for publication shortly before his death.

different compounds, all of which emit blue fluorescent light with only slightly different spectral band structures. Furthermore, all compounds are liable to be contaminated with fluorescent cell constituents. This excludes the sensitive but unspecific photoelectric comparison of the fluorescence intensities, and leaves, as the more reliable method, the match between a fluorescence spectrogram of a derivative under standard conditions, and the fluorescence spectrogram of the same derivative after its separation by the previously outlined method.

The matching may be done by photoelectric microphotometry or by direct visual comparison of the spectrograms. Although less sensitive, the visual method is the quicker. Furthermore, it has an added advantage in that the spectrograms usually exhibit a slight uniform background besides the normal bands, and the error due to this can be more easily reduced by visual examination than by microphotometry. The background itself can be reduced considerably by exciting the fluorescence, not with the strong ultraviolet Hg line at 365 m$\mu$, but with the weaker violet lines after filtering out the 365 m$\mu$ line. The use of the weaker violet lines does, however, involve considerable extension of exposure times to produce good spectrograms.

Apart from the uniform background which detracts from the specificity of the spectra, another serious source of error must be considered. Some preparations containing the X metabolites, especially those from mouse skins, exhibit on long exposures a band spectrum similar to that of the X metabolites. That this alien "S" spectrum—origin unknown—is due to some normal tissue component and not to any benzpyrene derivative is shown by the fact that it can be obtained from extracts of untreated skin. Instead of the almost uniform width of the 2-3 X bands, the violet "S" band is narrow and the "blue" band much wider. Any spectrogram showing this narrow violet band is useless for fluorimetric purposes. The system is therefore limited in its sensitivity to about 0.002 $\mu$g/c.cm by the presence of this unknown tissue component.

The method adopted was the visual matching of an unknown fluorescence spectrogram, exposed for time $t$ seconds with one of a series of fluorescence spectrograms of a standard solution of the same compound, at graded exposures to seconds. If the volume of the solution under test is $V$ cc. and the standard contains $c$ $\mu$gm./cc., the amount of metabolite is calculated as:

$$\frac{c \times V \times t}{E} \text{ gm.}$$

where $E$ is the maximal extinction.

For these results to be reliable, the fundamental conditions of fluorometry must be fulfilled; *i.e.*, the optical conditions must remain unchanged and the test solutions be so diluted that the fluorescent intensity be practically uniform throughout the whole layer of liquid.

The standard solutions of known concentration $c$ were made up from preparations obtained by chromatography, as described in Part I of this series (2). As the chemical identities of the derivatives are unknown, the estimations of the standard solutions have been done by absorption spectro-photometry. If volume $V$ of the solution is used in a tube length $L$, and $E$ is the maximal extinction in the near ultraviolet whilst $C$ is a special constant for each derivative, the amount, $N$, present in the solution is given by the expression:

$$N = \frac{V E}{L C} \text{ mgm.}$$

The numerical values of $C$ were the same as previously adopted (2); *i.e.* for benzpyrene 120 at 385 m$\mu$, for $X_1$ and $X_2$, 85 at 380 m$\mu$ and for $F_1$ and $F_2$, 100 at 380 m$\mu$. Additionally, the two maxima in the violet may be used for the separate estimation of $F_1$ and $F_2$. In this case, the values for $C$ are: $F_1$=65 at 118 m$\mu$, and $F_2$=40 at 425 m$\mu$.

This last equation may be used in cases where the less sensitive but more direct absorption spectrophotometric method is applicable. Examples of such cases are:

The estimation of the sum of the $X_1$ and $X_2$ metabolites in the extracts of intestines prior to separation.

The total estimation of $F_1$ and $F_2$ in xylene extracts of feces, when negligible amounts of unchanged benzpyrene are present.

The method of absorption spectrophotometry is generally useful for the estimation of residual, unchanged benzpyrene in the carcass. But in all cases where the amounts involved are too small for absorption spectophotometry recourse must be made to the fluorimetric method.

## DISCUSSION

The methods developed for the quantitative estimation of the various intermediate products of the metabolic conversion of benzpyrene are the result of a number of compromises. Two main factors limit the systems:

The necessity to use physical methods for the separation of the metabolites—imposed by the ignorance of the exact chemical nature of the substances in question.

The fact that the benzpyrene derivatives are rela-

tively unstable and can, therefore, be obtained only in small quantities.

Both of these factors reduce the precision of the methods. Nevertheless, the order of quantitative results obtained under these conditions must be regarded as reliable and as such is of value in the general approach to the problem of the metabolism of benzpyrene.

## SUMMARY

1. An improved technic, founded upon physical properties of the substances concerned, for the separation of the metabolic products of 3,4-benzpyrene is described.

2. A fluorimetric method for the quantitative estimation of these products is outlined.

3. Consideration is given to the conditions under which absorption spectrophotometry can be used for quantitative estimations.

4. The limitations of the systems considered are discussed.

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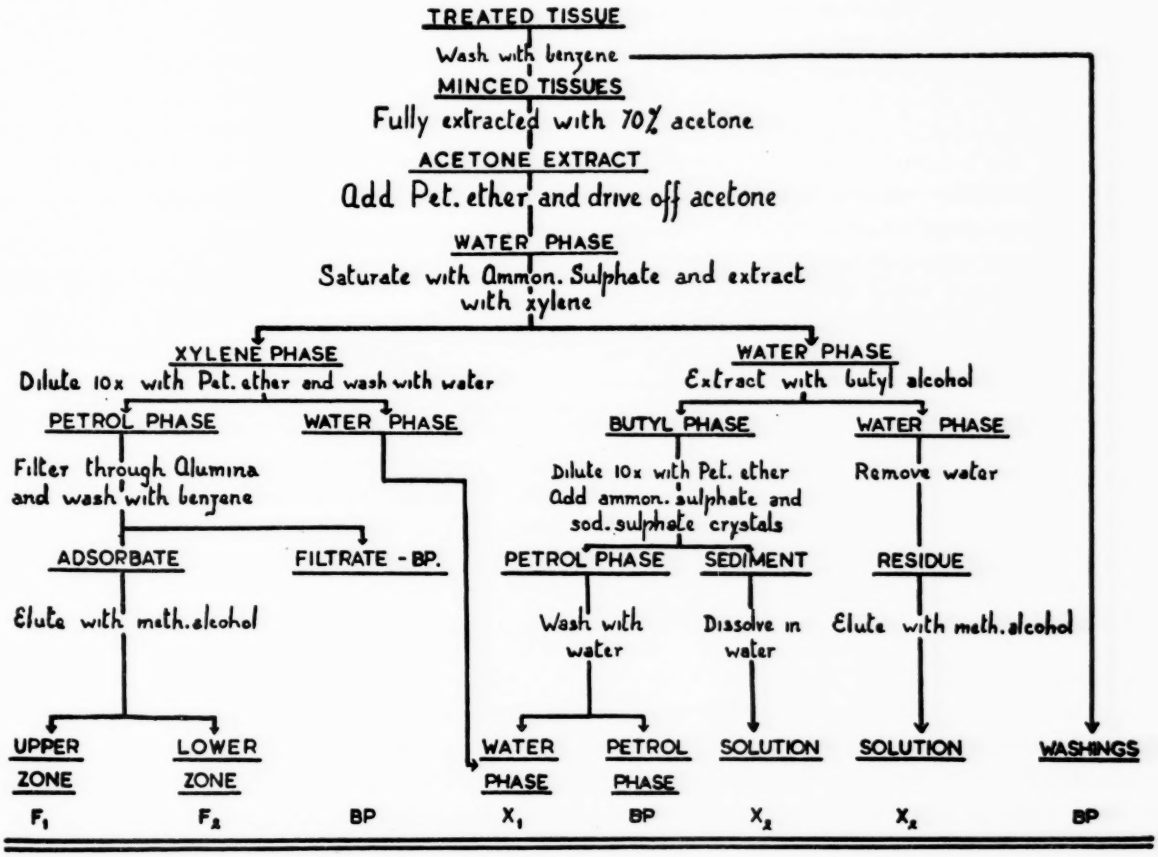


Fig. 1.—Chart showing method of extraction of metabolites. Decision as to whether the whole or part of the scheme should be used must depend on the experiment in hand.

# Prothrombin Activity in Rats with Mammary Tumors*

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The status of hepatic function can be altered by diverse influences. Multiple functional processes are measurably affected by several extrinsic toxic agents (typhoid toxin, chloroform, yellow phosphorus) as well as by a large number of pathological disorders. After exposure to toxins, in obstructive jaundice, in cirrhotic processes, in intrahepatic tumors and the like, either anatomical disruption or functional impairment may first be observed, but generally the two are inseparable.

We have recently reported that only after extensive replacement of normal liver tissue by large hepatic tumors induced in rats by *p*-dimethylaminoazobenzene is the normal capacity of the liver to elaborate prothrombin reduced (4). This condition is not corrected by vitamin K. Furthermore, the anticoagulant, 3,3′-methylenebis (4-hydroxycoumarin),[1] caused a more severe and persistent hypoprothrombinemia in rats with primary hepatic tumors than in normal rats. The purpose of the present report is to indicate that a gross increase in normal hepatic tissue has been observed in rats with spontaneous mammary tumors and that this condition is associated with a greater functional reserve as evidenced by increased plasma prothrombin activity and a reduced hypoprothrombinemic response to a standard dose of 3,3′-methylenebis (4-hydroxycoumarin).

## METHODS

The basic technics employed as well as the rationale of handling the animals are given in previous publications (4, 11). The rats were fed the semi-synthetic diet for at least 6 days prior to the initial determination of prothrombin activity. They were fasted for 12 hours and fed 2.5 mgm. of 3,3′-methylenebis (4-hydroxycoumarin) contained in 2 gm. of food. Twenty-four hours after the anticoagulant was consumed, blood samples were taken by heart puncture under light ether anesthesia. The clotting time of 12.5 per cent plasma was determined by our standard procedure (1). Determinations of prothrombin activity were routinely made on whole plasma. However, since any changes in prothrombin activity[2] are readily detected and reproducibly determined by the diluted plasma technic, and are frequently obscured in the whole plasma determination by the inherent relatively large technical variation, the presentation of data is restricted to results obtained with 12.5 per cent of plasma.

*Tumors.*—Healthy rats with grossly visible spontaneous mammary tumors in various stages of development were provided in generous numbers from the stock colonies of Sprague-Dawley, Inc., Madison. Some confirmatory trials and analyses were performed on rats of the Wistar strain with spontaneous mammary tumors raised in the stock colony of the Rochester laboratories. In every case the tumor was examined by gross dissection, and sections stained with hematoxylin-eosin were classified by microscopic survey. The tumors were single or multiple, and in some rats the multiple tumors arose from widely separated mammae. They were discrete, well encapsulated, and varied in diameter from 2 to 8 cm. Characteristically, they showed no evidence of penetration into subcutaneous tissues. Predominantly, they were of the dense fibroadenoma variety with a minimum of duct and acinar proliferation amongst a diffuse connective tissue

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This paper represents a phase of the studies on the anticoagulant 3,3′-methylenebis (4-hydroxycoumarin) carried out in the laboratory of Prof. Karl Paul Link (see the Harvey Lectures Series XXXIX, 162-216. 1943-1944. The author wishes to acknowledge his indebtedness to Professors Link and C. A. Baumann for their counsel and guidance.

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[1] Available to the clinician under the trademark *Dicumarol.*

[2] The effectiveness of the diluted plasma technic in revealing increased prothrombin activity (hyperprothrombinemia) has been discussed in Field, J. B., Larsen, E. G., Spero, L. and Link, K. P. Studies on the Hemorrhagic Sweet Clover Disease XIV. Hyperprothrombinemia Induced by Methylxanthines and its Effect on the Action of 3,3′-Methylenebis (4-hydroxycoumarin). J. Biol. Chem. 156, 725-737 (1944).

matrix. There was no histological evidence of malignancy, degeneration, or edema.

## EXPERIMENTAL

*Prothrombin activity of plasma from normal and tumor-bearing rats.*—The prothrombin time of 12.5 per cent plasma from normal rats ranged from 34 to 46 seconds, average 39 (Table I). Only 4 of 360 samples (0.9 per cent) clotted in less than 35 seconds. The prothrombin time of the plasma from rats with mammary tumors ranged from 31 to 41 seconds, average 35. Of 38 samples from tumorous rats, 17 (44 per cent) clotted in less than 35 seconds. It has been repeatedly emphasized that this decrease in average clotting time probably represents an appreciable increase in prothrombin since in this particular time range of the plasma (prothrombin) dilution curve, a large change in prothrombin concentration produces a relatively small change in the clotting time[3] (1).

TABLE I: PROTHROMBIN TIME OF 12.5 PER CENT PLASMA FROM NORMAL RATS AND RATS BEARING MAMMARY TUMORS

| | Prothrombin time Average | Range seconds | No. of rats | Animals below normal, per cent* |
|---|---|---|---|---|
| Normal rats | 39 | 34–46 | 360 | 0.9 |
| Rats bearing spontaneous mammary tumors | 35 | 31–41 | 38 | 44.0 |

* This lower limit of "normal" plasma was arbitrarily considered to be 35 seconds.

The mere presence of a neoplasm in the animal does not of itself affect the prothrombin levels (4).

*Induced hypoprothrombinemia.*—Twenty-four hours after the administration of 2.5 mgm. of the anticoagulant a definite and readily measured hypoprothrombinemia was observed in all normal rats (Table II). However, the hypoprothrombinemia induced by the anticoagulant in rats with mammary tumors was neither extensive nor consistent. The average prothrombin time of plasma from normal rats was 116 seconds with 22 of 463 samples (4.8 per cent) showing a prothrombin time of less than 85 seconds. In contrast, the prothrombin time from rats with mammary tumors averaged 80 seconds with samples from 21 of 29 rats (72.4 per cent) giving prothrombin times of less than 85 seconds.

TABLE II: INDUCED HYPOPROTHROMBINEMIA IN NORMAL RATS AND RATS BEARING MAMMARY TUMORS

Prothrombin time of 12.5 per cent plasma 24 hours after the administration of 2.5 mgm. of 3, 3′-methylenebis (4-hydroxycoumarin).

| | Prothrombin time Average | Range seconds | No. of rats | Animals below normal, per cent* |
|---|---|---|---|---|
| Normal rats | 116 | 78–215 | 463 | 4.8 |
| Rats bearing spontaneous mammary tumors | 80 | 50–135 | 29 | 72.4 |

* The lower limit for "normal animals" receiving this dosage of anticoagulant was arbitrarily considered to be 85 seconds.

There was a wide variation in the degree of hypoprothrombinemia that the standard dose of anticoagulant induced in individual rats with mammary tumors. But in most of the tumorous rats the hypoprothrombinemic response obtained from testing with a standard dose of anticoagulant after successive resting periods of 2 to 3 weeks was progressively reduced. Thus a typical illustration of the increasing resistance to induced hypoprothrombinemia taken from the protocol of a rat with a medium sized tumor over an 11 week period is 113, 89, 80 seconds. However, there was no fixed correlation between the size or number of developing mammary tumors and the resistance to induced hypoprothrombinemia. In some rats with relatively large tumors a progressive debilitation in general health tended to interfere with such resistance and to increase the degree of hypothrombinemia.

*Fibrinogen levels of tumorous rats.*—Deutsch and Gerarde (3) have recently indicated their belief that the hyperprothrombinemic states, indicated in previous reports from this laboratory (6), reflect primary elevation in fibrinogen levels. The latter condition is usually elicited by the methylxanthine drugs (8) and only rarely by vitamin K (5). However, the fibrinogen levels of rats with mammary tumors as determined by standard procedures (9, 13) averaged 183 mgm. per cent, compared to an average of 274 mgm. per cent for non-tumorous control rats.

*Effect of surgical excision of tumors.*—After the individual prothrombin activity and degree of induced hypoprothrombinemia had been well established in rats with 1 or 2 mammary tumors, the latter were excised. In a brief manipulation under ether anesthesia accomplished with little bleeding, the well encapsulated masses were readily identified and removed. Repeated determinations of prothrombin activity and the hypoprothrombinemia induced by the anticoagulant were made at intervals of 7 to 10 days. A summary of data from animals surviving 61 days postoperatively and from whom several repeated values were obtained, is given in Table III. Whereas the prothrombin time of 12.5 per cent plasma of this group of rats was 34 seconds in the presence of the tumor, the prothrombin time was 38 seconds following excision of the tumor (compared to an average of 39 seconds in non-tumorous normal rats). The prothrombin time of 12.5 per cent plasma 24 hours after a stand-

[3] Field, J. B., Scheel, L., Baumann, C. A., and Link, K. P. Unpublished data.

ard dose of the anticoagulant was 70 seconds prior to the operation and following excision averaged 106 seconds (compared with 116 seconds in normal rats). The response to the anticoagulant immediately after excising the tumor was often temporarily irregular for 2 to 4 weeks, but ultimately the degree of hypoprothrombinemia induced in 13 of 15 rats was that characteristic of non-tumorous rats. The hypoprothrombinemia elicited in 2 of the rats indicated a persisting resistance for 9 weeks postoperatively.

*Liver and pituitary weights.*—A survey was made of the weights of pertinent organs from healthy rats 23 mgm. As indicated in Table IV, there was a rough correlation between the weight of the mammary tumor and the size of the liver and pituitary gland. In animals possessing more than one tumor the pertinent organ weights resembled those from animals with a single tumor of the same weight as the weight of the combined tumors. Following excision of the tumors the liver and pituitary weights apparently regressed to values characteristic of the control females (Table III). At death, the livers averaged only 2.5 per cent (fresh weight) of the body weight while the pituitary gland averaged 14.4 mgm.

TABLE III: PROTHROMBIN TIME AND INDUCED HYPOPROTHROMBINEMIA IN RATS BEARING MAMMARY TUMORS BEFORE AND AFTER SURGICAL EXCISION OF THE TUMORS*

| | Normal prothrombin time (12.5 per cent plasma) (in seconds) | | Induced Hypoprothrombinemia in 12.5 per cent plasma (in seconds) | | Weight of tumor at removal, gm. | At death | | | |
|---|---|---|---|---|---|---|---|---|---|
| | With tumor | After excision | With tumor | After excision | | Body, gm. | Liver, gm. | Liver as per cent body weight | Pituitary, mgm. |
| Average | 34 | 38 | 70 | 106 | 41 | 397 | 10.1 | 2.54 | 14.4 |
| Range | 32–37 | 35–41 | 56–113 | 67–165 | 22–68 | 361–413 | 8.3–12.5 | | 12.4–17.7 |

* Of a large group of rats with medium to large mammary tumors, only 6 survived to provide successive values incorporated in the above prothrombin data. Following operative excision of the tumors the rats were subjected to periods of induced hypoprothrombinemia and frequent cardiac punctures. The survival times of the rats used to compile this summary, dating from the operation, was from 14 to 61 days.

TABLE IV: LIVER AND PITUITARY SIZE IN NORMAL RATS AND RATS BEARING MAMMARY TUMORS

| | Fresh tumor weight† + S.D., gm. | Body weight + S.D., gm. | Fresh liver weight + S.D., gm. | Liver as % of body weight | Fresh pituitary weight + S.D., mgm. | Number |
|---|---|---|---|---|---|---|
| Normal rats | ..... | 273 ± 13.0 | 8.8 ± 0.9 | 3.22 | 11.8 ± 0.9 | 25 |
| | ..... | 345 ± 20.6 | 9.8 ± 1.3 | 2.84 | 13.9 ± 1.6 | 18 |
| Rats bearing spontaneous mammary tumors | 6.0 ± 2.2 | 353 ± 33.0 | 12.4 ± 1.3 | 3.57* | 14.4 ± 1.8 | 13 |
| Single | 19.3 ± 5.4 | 364 ± 30.0 | 14.6 ± 2.9 | 4.23* | 15.6 ± 2.3 | 30 |
| | 53.2 ± 25.5 | 395 ± 29.4 | 14.9 ± 2.9 | 4.36* | 15.1 ± 2.6 | 22 |
| (Multiple**) | 24.9 ± 15.7 | 371 ± 30.2 | 14.8 ± 3.0 | 4.28* | 16.6 ± 1.2 | 5 |

S.D. Standard deviation.
† Arbitrarily classified by tumor size.
* Calculated on the net body weight minus tumor. When the gross body and tumor weight is applied as the divisor, these values range from 3.51 per cent to 4.01 per cent.
** In these animals 2 to 3 isolated and separate tumors were found. The weight given is that of the total of all tumors found.

bearing spontaneous mammary tumors. The summary of the weights of the tumor, liver and pituitary gland and the weights of the liver and pituitary gland obtained from healthy non-pregnant non-lactating stock female rats of comparable body weight is given in Table IV. The liver (fresh weight) is approximately 2.9 per cent of the body weight of large normal non-tumorous female rats. However, the ratio in rats of comparable body weight with mammary tumors ranged from 3.6 to 4.4 per cent. Similarly the weight of the pituitary gland in normal non-tumorous rats is approximately 13.9 mgm., whereas in rats with mammary tumors the gland weighed from 14.4 to 16.6 mgm. The largest specimens in each category were (a) a healthy normal female of 346 gm. with a liver of 12.2 gm. (3.5 per cent) and a pituitary gland of 15 mgm. and (b) a rat of 387 gm. with a tumor weighing 10.3 gm. which had a liver weighing 23.3 gm. (6.1 per cent of body weight) and a pituitary gland weighing

*Microscopic and chemical examination of livers.*—Samples of liver from sacrificed rats bearing mammary tumors were placed in Bouin's fixative and stained in routine fashion with hematoxylin and eosin. A careful microscopic examination of sections from a large series of tumorous rats was made. In every case the liver cells were regular in shape and content and stained in the usual manner. No increase was observed in mitotic figures. The hepatic sinusoids were somewhat prominent and generally an increased vascularity was apparent, but there was no congestion.

Total liver lipids were determined by grinding with sand and extracting with ether and chloroform, combining the extractives and evaporating the solvents. Total moisture of the livers was determined by drying liver slices at 100° F. for 48 hours. The content of total liver lipids and moisture from normal and tumorous animals were similar.

## DISCUSSION

A reduction in the number of functioning liver cells has previously been shown to result in a plasma that is mildly hypoprothrombinemic and especially sensitive to the hypoprothrombinemic action of the anticoagulant, 3,3′-methylenebis (4-hydroxycoumarin) (4). A significant increase in liver size was associated with observations of a marked resistance to induced hypoprothrombinemia detectable in normal lactating rats (7). The observations described in the present study are in harmony with the assumption that an increase in the number of normal liver cells produces a state of increased plasma prothrombin activity and a reduced hypoprothrombinemic response to a standard dose of the anticoagulant.

It is not improbable that the hepatomegaly arising during the development of a mammary tumor is associated with, or secondary to, what appears to be a true hypertrophy of the pituitary gland. It may be presumed that the pituitary hypertrophy connotes hyperfunction and in the absence of more objective evidence, the increase of the pituitary weight in the tumorous state is offered as a *prima-facie* evidence of this rationalization. The nature, if any, of the relationship between the developing mammary tumor and the pituitary gland is an object for further study. However, a relationship between the liver and the pituitary is on a firmer basis. Hypophysectomy has been shown to produce a reduction in liver weight in the pigeon (14) and rat (15) and hepatic cirrhosis in the dog (10), and conversely acromegaly (pituitary hyperfunction) in the dog (12) and man (2) have been associated with marked increases in liver size. At least 2 individual hormonal preparations in the hands of several investigators, prolactin and the growth factor, have induced splanchnomegaly and particularly hepatomegaly (10, 14). When purified prolactin preparations were administered parenterally to rats and rabbits, there developed a tendency to increased plasma prothrombin activity and more generally a reduced hypoprothrombinemic response to a standard dose of the anticoagulant.[3] These data were in agreement with those obtained from lactating rats (7) and are consistent with those contained in the present report.

The excess of functional liver tissue apparently imparts to the lactating rat, and rat with a mammary tumor, an enhanced capacity to recover from incipient or induced hypoprothrombinemia. Undoubtedly prothrombin is only one product of liver metabolism increased above normal in liver tissue, and it is possible that further studies will reveal other substances similarly affected. However, it cannot be assumed that a gross hepatic hyperplasia and consequent increased hepatic function fortifies the animal against all manner of hepatotoxins. In fact, in separate trials we have been unable to demonstrate that the susceptibility of the livers of healthy rats with mammary tumors given chloroform is less than those of non-tumorous rats.[4] It is surprising that an increased hepatic reserve does not protect the rat against the lethal action of a hepatotoxin such as chloroform.

Independent observations by Mr. Lester Scheel in Prof. Link's laboratory during the summer of 1947 confirm the findings recorded here. In 15 rats with mammary tumors, the average clotting time of 12.5 per cent plasma was 32.9 $\pm$ 2.9 seconds compared with a clotting time in non-tumorous female rats of 40 $\pm$ 2 seconds. The 6.25 per cent diluted plasma from the tumor rats was 33.2 $\pm$ 3 seconds compared with the 47.3 $\pm$ seconds of control rats. The increase in "clotting activity" of the plasma of mammary tumor rats was also apparent when 0.3 per cent fibrinogen solution was employed as diluent. Finally, the mean of the plasma fibrinogen of the tumorous rats was 267 mgm. per cent compared with a value of 300 mgm. per cent in normal rats.

## SUMMARY

1. The presence in the rat of spontaneous mammary tumors causes a reduction of the normal prothrombin time (12.5 per cent plasma).

2. The hypoprothrombinemia induced by a standard dose (2.5 mgm.) of the anticoagulant, 3,3′-methylenebis (4-hydroxycoumarin), is considerably less in rats with mammary tumors than in non-tumorous controls.

3. The presence of mammary tumors in rats appears to be associated with an increase in the size of the liver and pituitary gland. There is some correlation between the weight of the mammary tumor and weight of liver and pituitary gland. The hyperplastic liver is composed of microscopically normal hepatic cells and the moisture and total lipids of the liver are within normal limits.

4. Following excision of the mammary tumor, the prothrombin activity and the extent of induced hypoprothrombinemia increased to levels resembling those obtained from normal rats. Similarly, the weights of the liver and pituitary gland were similar to those of non-tumorous rats.

5. The hepatomegaly and prothrombin response observed in rats with mammary tumors is similar to data obtained from lactating rats and rats treated with the hormone, prolactin. Rats with mammary

[4] Field, J. B. Unpublished data.

tumors are not less susceptible to the hepatotoxic and lethal action of chloroform.

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# The Effect of Repeated Applications of Minute Quantities of Mustard Gas ($\beta\beta'$-Dichlorodiethylsulphide) on the Skin of Mice*

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## INTRODUCTION

In earlier experiments, Fell and Allsopp (4) found that when low concentrations (0.05 mgm./cc.) of mustard gas are added to the nutritive medium of tissue cultures *in vitro,* the cells continue to divide but many do so abnormally forming multinucleate, hypertrophic and other atypical cells similar to those seen in malignant tumors.

In view of these observations it was decided to study the histological changes produced in the skin of mice by the repeated application of small doses of mustard gas with particular reference to the effect of such treatment on the epidermis (5).

## MATERIAL AND METHODS

Two series of mice were used, the first (11 mice) being treated with a solution of 250 $\gamma$ / cc. mustard gas in acetone and the second (10 mice) with a solution containing 50 $\gamma$ / cc. mustard gas. The mice were painted about five times a week by dropping 0.05 cc. of the solution on the back with a calibrated pipette.

Two mice in each series died during the course of the experiment and, being unfit for histological study, were rejected. The rest died or were killed at different intervals (Table I) and the painted area of the skin was excised, fixed and sectioned. In most of the animals half the treated skin was fixed in acetic Zenker's fluid and sections were stained with azan, Ehrlich's hematoxylin and erythrosin or (series 1) Feulgen's method, while the other half was fixed in 80 per cent alcohol and stained by Gömöri's method for the demonstration of alkaline phosphatase (9). The latter preparations were incubated for 20 to 24 hours in the glycerophosphate solution instead of the 2 hours recommended by Gömöri, as previous experiments had shown that for scar tissue better results were obtained with the longer incubation (3, 6).

## RESULTS

### Series I

*Observations on the living mice.*—A few days after treatment had begun, the painted area became irritated. After 14 days large incrustations had developed. These were sloughed, carrying the hairs with them and leaving a smooth healed surface. By the end of the third week the skin was edematous and sometimes ulcerated.[1]

By the 30th day the hair was growing again on the treated area which showed also ulcerating patches and sloughing keratin. Epilation was repeated about the 56th day but 16 days later the hair was again growing strongly. It was shed a third time about the 130th day and was not regenerated on the painted area until after treatment ended on the 271st day.

After the paintings ceased the skin began to heal and hair grew over the whole site after all the keratin had sloughed. This growth of hair, however, was thinner than usual and the painted area could easily be identified until the end of the experiment.

No sign of tumor formation was seen at any stage of the experiment.

*Histological observations.*—The skin fixed after 2 days' painting (Table I, No. 1) showed a very slight leukocytic infiltration of the dermis but no other change. That fixed after 32 days (No. 2) had a very thick keratin layer and an abnormally dense dermis with a fairly heavy leukocytic infiltration; unfortunately post-mortem changes prevented more detailed examination as the animal had died during the night.

The skin painted for 66 days (No. 3) varied in

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NOTE: Because of accelerated production schedule, author has not read proof of this paper.

[1] When serious ulceration occurred, painting was suspended for 2 or 3 days.

structure in different regions. In places the epidermis had disappeared and was replaced by a thick scab, elsewhere it appeared attenuated and atrophic but in other parts it was greatly thickened and proliferated actively. The cells of the growing region looked almost normal but the hair follicles were very atypical and many were hyperkeratotic (as stated above, epilation had occurred a few days before). Ulceration had reached the fatty layer.

The painted skins of 4 animals killed on the 273rd day (Nos. 4-7) were very abnormal. In places the epithelium was greatly thickened (Fig. 1) and contained many atypical cells similar to those present in cultures grown in medium containing low concentrations of mustard gas (4). Multinucleate cells (Fig. 2) with nuclei of widely varying size were very common and all stages in their development from abnormal mitosis were seen. Hypertrophic nuclei (Fig. 3) and nuclei with enormously enlarged plasmasomes were also numerous. A large proportion of the mitotic figures were abnormal; clumping of the chromosomes, chromosome lag (Fig. 4) and more rarely polyploid cells (Fig. 5) and multipolar division were encountered. Somewhat similar abnormalities were observed by Gillette and Bodenstein in tadpoles grown in solutions of the "nitrogen mustard," methyl-bis-($\beta$-chlorethyl)-amine (7), and by Koller, Ansari and Robson (10, 11) in the mitotic cells of *Tradescantia* treated with mustard gas. In 3 mice there were areas of active epithelial downgrowth into the dermis, but these downgrowths were sharply defined and the cells fairly well differentiated. Many hair follicles were abnormal. As in No. 3, in some regions the epidermis seemed partly atrophied and consisted of a few enlarged cells only, while elsewhere it had been shed and replaced by a scab. Hyperkeratosis was seen in all four mice. The dermis was abnormally thick and dense beneath the actively proliferating epithelium and was composed mainly of young collagen fibers, but in the denuded areas there was very little fibrous material and ulceration extended to the fatty layer.

Part of the painted skin in each of these 4 mice had been treated by Gömöri's method for the demonstration of alkaline phosphatase. The ulcerating areas differed in a curious way from ordinary wounds and heat burns in similar preparations. In the latter the scab appears nearly black and is always the most intensely reacting part of the section; this is due to its high content of polymorph leukocytes which contain much phosphatase (6). The scabs on the mustard-painted skins on the other hand gave almost no reaction although heavily infiltrated with polymorphs. The epidermis and superficial layers of the dermis were also nearly free of phosphatase but below this zone the dermis reacted strongly, although in uninjured skin it contains little or no phosphatase. In healing wounds and heat burns a strong reaction always accompanies the regeneration of collagen fibers (3, 6) and a similar correlation between fiber-formation and phosphatase activity seemed to exist in the mustard gas lesions.

TABLE I: EXPERIMENTAL MATERIAL

| Series | Exp. no. of mouse | Duration of painting | Approx. total dose | Survival after end of painting | Total period of survival (from beginning of painting) |
|---|---|---|---|---|---|
| 1 | 1 | 2 days | 25$\gamma$ | .... | 2 days |
| | 2 | 32 " | 275$\gamma$ | .... | 32 " |
| | 3 | 66 " | 550$\gamma$ | .... | 66 " |
| | 4 | 271 " | 2000$\gamma$ | 2 days | 273 " |
| | 5 | " " | " | " | " " |
| | 6 | " " | " | " | " " |
| | 7 | " " | " | " | " " |
| | 8 | " " | " | 79 days | 350 " |
| | 9 | " " | " | 100 " | 371 " |
| 2 | 1 | 130 days | 200$\gamma$ | .... | 130 days |
| | 2 | 278 " | 450$\gamma$ | 2 days | 280 " |
| | 3 | " " | " | " | " " |
| | 4 | " " | " | " | " " |
| | 5 | " " | " | " | " " |
| | 6 | " " | " | 65 days | 343 " |
| | 7 | " " | " | 93 " | 371 " |
| | 8 | " " | " | 93 " | 371 " |

Healing was well advanced in a painted area fixed 79 days after the end of treatment (No. 8). The epidermis was still slightly thickened in places, though no abnormal cells and very little mitosis were seen, and many of the hair follicles were cystic. The dermis was rather more dense than usual but the strong phosphatase reaction seen at the previous stage had nearly disappeared, as in a fully healed wound.

In a mouse killed 100 days after the end of painting (No. 9) there was a slight local thickening of the epidermis, some hyperkeratosis and a reduction in the normal number of hair follicles. The dermis seemed normal and gave little phosphatase reaction.

### Series 2

*Observations on the living mice.*—Epilation first appeared rather later than in Series 1, *i.e.*, after 3 weeks' painting. Hair began to regenerate about the 38th day and was again being shed by the 62nd day. The treated skin was ulcerated much less than in Series 1, but appeared to be irritable. There was no actual tumor formation. In the earlier stages, 2 or 3 mice developed thickenings which resembled incipient tumors, but these regressed and were probably areas of hyperkeratosis.

*Histological observations.*—The epidermis of the mice examined during (No. 1) and two days after (Nos. 2-5) painting was abnormally thick but proliferation was much less active than in the equivalent mice of Series 1. The cells were somewhat enlarged and those of the basal layer, which showed very little mitosis, were mostly transformed into prickle cells. Abnormal mitotic figures and multinucleate cells occurred but were much less numerous than in Series 1. The hair follicles were usually hyperkeratotic or cystic. The dermis contained many fine, newly formed collagen fibers and was fairly heavily infiltrated with leukocytes.

The distribution of phosphatase resembled that in the skin of Series 1, *i.e.*, the reaction was most intense in the regenerating fibrous tissue and the proximal ends of the hair follicles, while the scabs and the most superficial part of the tissue contained very little phosphatase.

## DISCUSSION

The results of our experiments have shown that the repeated application of small doses of mustard gas over long periods has a very pronounced effect on the epidermal nuclei, the cytological abnormalities produced resembling those previously seen in fibroblasts cultivated in medium containing low concentrations of mustard gas (4). On the other hand, differentiation is not affected in the regenerating epidermis, a result which agrees with that of Gillette and Bodenstein (7) who found that in tadpoles treated with a nitrogen mustard compound "the agent selectively affects mitosis and not differentiation."

It is interesting to compare the histological changes in the skin of our experimental animals with those appearing in mice treated with 3,4-benzpyrene (8). Both treatments produced a cumulative effect, an alternation of degeneration and repair indicated macroscopically by alternating epilation and regeneration of hair, and nuclear disturbances manifested in abnormal mitosis, multinucleate cells, hypertrophic nuclei, etc.

There are, however, important differences between the effects of the two substances. Painting with dilute mustard gas has a more drastic nuclear action than painting with benzpyrene and causes a much larger proportion of severe cell abnormalities. In the skins treated with mustard gas, the hyperplastic regions are usually near an ulcerating area and the cell proliferation appears to be no greater than is required for the replacement of the damaged tissue. When epithelial downgrowth into the dermis occurs, the downgrowing processes have a sharply defined outline and do not show that excessive proliferation of the undifferentiated basal cells followed by diffuse invasion of the surrounding tissue which characterizes skins treated with benzpyrene. The fundamental difference between the histological effects of treatment with dilute mustard gas and with benzpyrene seems to be that while the former disturbs mitosis but permits differentiation, the latter stimulates mitosis but inhibits differentiation.

This difference may explain why, under the conditions of our experiments, no tumors were formed in response to the mustard gas. Many more animals would have to be treated before the non-carcinogenicity of the agent could be finally established, but our results agree with those of Berenblum (1, 2). Working with experimental conditions that differed from ours in some respects, he not only obtained no tumors in mice by the repeated application to the skin of small quantities of mustard gas, but also made the important observation that similar amounts of the agent inhibit the carcinogenic action of tar on the skin.

There is an interesting similarity between the histological effects of the repeated application of small quantities of mustard gas and of a single massive dose of x-rays. Glucksmann (unpublished results) has recently shown that such an irradiation produces mitotic abnormalities in the skin of

mice but does not interfere with differentiation or cause tumors. These observations further emphasize the fact that although abnormal mitosis is a usual feature of malignant tissue it is not a specific character of malignancy.

The effect of repeated applications of dilute mustard gas on the alkaline phosphatase of the skin is interesting. The mustard gas appears to destroy or inactivate the phosphatase in the scab, which in wounds contains a large concentration of the enzyme, but this inhibitory effect does not seem to penetrate very deeply as the regenerating fibrous tissue of the dermis reacts strongly as it does in a healing wound or heat burn.

## SUMMARY

1. Experiments were made to investigate the histological effect on the skin of mice of the repeated application of small doses of mustard gas (12.5 $\gamma$ and 2.5 $\gamma$).

2. The following similarities between skin treated with small doses of mustard gas and that treated with 3,4-benzpyrene were observed: the effect of the treatment was cumulative; there was a repeated alternation of degeneration and repair; nuclear abnormalities (abnormal mitosis, multinucleate cells, hypertrophic nuclei, etc.) were produced in the epidermis; in certain areas the epidermis became hyperplastic.

3. The effect of small doses of mustard gas differed from that of 3,4-benzpyrene as follows: no tumors developed under the conditions of our experiments; the nuclear disturbance was more drastic causing a much greater proportion of cell abnormalities; the hyperplastic regions of the epidermis were much more differentiated and showed no sign of the diffuse invasion of the connective tissue which characterizes malignancy; cell proliferation appeared to be no greater than was required to replace the damaged tissue.

4. The effect resembled that of a single massive dose of x-rays.

5. Preparations of the skins treated with mustard gas were made by Gömöri's method for the demonstration of alkaline phosphatase. The scab and immediately subjacent tissue gave no reaction, but the regenerating dermal tissue reacted strongly (in heat burns the scab reacts even more intensely than the regenerating connective tissue; the normal dermis has little or no phosphatase).

6. The treated areas had largely recovered in mice examined 65 to 100 days after cessation of painting, but the skin was not quite normal.

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## DESCRIPTION OF FIGURES 1 TO 5

The photomicrographs were made by Mr. V. C. Norfield, Head Assistant at the Strangeways Research Laboratory.

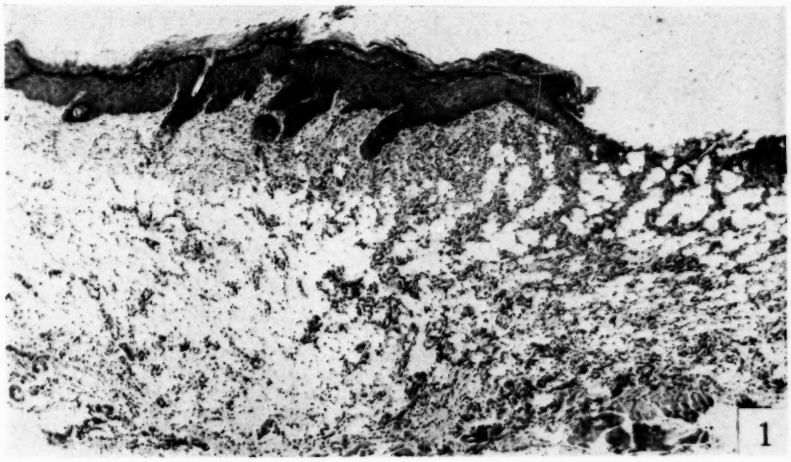
Fig. 1.—Section of the skin of a mouse from Series 1 killed on the 273rd day (*i.e.*, 2 days after the end of treatment) showing the greatly thickened epidermis adjacent to an ulcerating area. Hematoxylin and erythrosin stain. Mag. × 40.

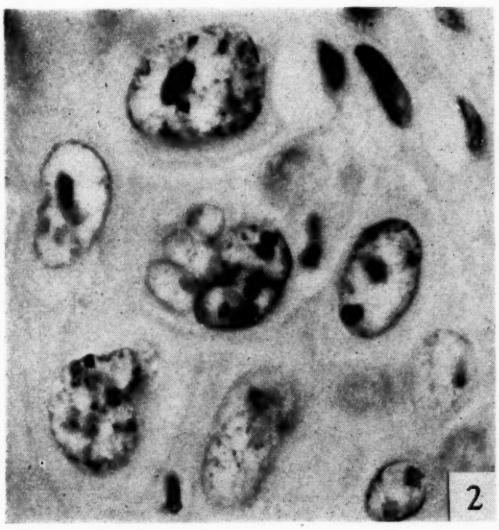
Fig. 2.—Section of the skin of the same mouse showing a multinucleate cell and several hypertrophic cells in the epidermis. Feulgen stain. Mag. × 900.

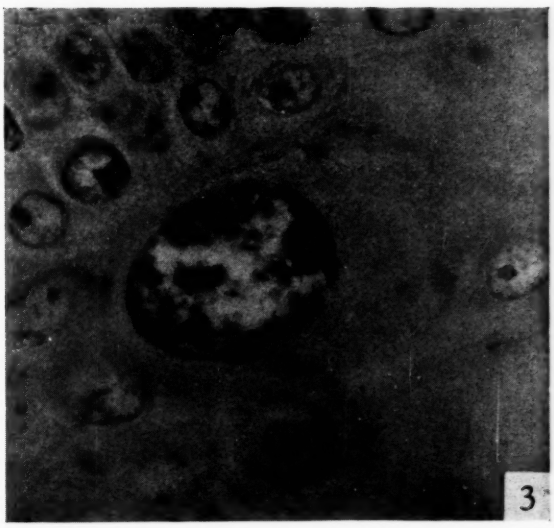
Fig. 3.—Section of the skin of another mouse from the same series killed on the 273rd day showing a hypertrophic epidermal cell. Feulgen stain. Mag. × 900.

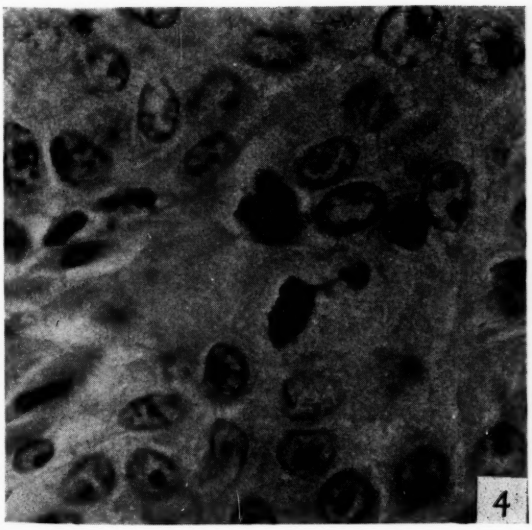
Fig. 4.—An abnormal telophase with chromosome "lag" in the same skin as that shown in Fig. 3. Feulgen stain. Mag. × 900.

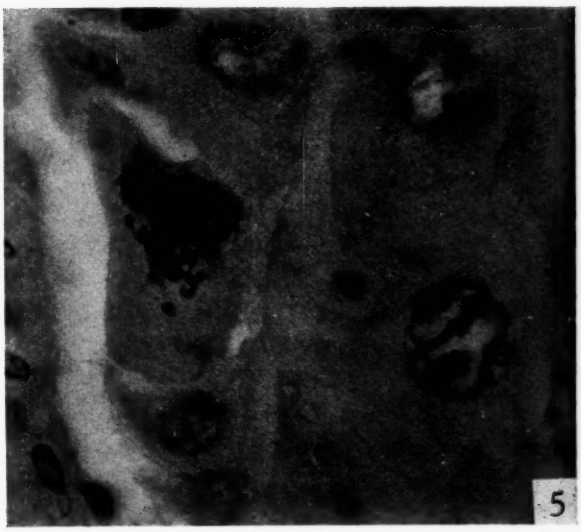
Fig. 5.—Polypoid cells in the same skin. A multinucleate cell is also seen. Feulgen stain. Mag. × 900.











Figs. 1–5

# Retardation of Growth of a Transplantable Carcinoma in Mice Fed Basic Metachromatic Dyes

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Some years ago an experiment was briefly reported (25) in which it was found that a single subcutaneous injection of 0.1 cc. of heparin (Vitrum, Stockholm) into mice at the expected time of emergence of 1, 2, 5, 6- dibenzanthracene-induced papillomas not infrequently led to the localization of a tumor in a small elliptical wound placed over the wheal raised by the injection in the painted area. Of 47 mice surviving this treatment, 8 carcinomas appeared in the scars; while in a further 11 animals the wounds in course of healing showed hyperkeratosis or small papillomas which subsequently regressed. No tumors arose from the scars of a comparable group of mice in which chick embryo extract replaced the heparin. Heparin (34) and embryo extract (4) were chosen for these experiments on account of their reported effects in causing, respectively, inhibition and stimulation of the growth of normal cells in tissue culture.

The mechanism whereby heparin exerts an inhibitory effect on cells in tissue culture is still obscure, though recent work by Fischer (10) suggests that heparin or similar colloidal compounds carrying a strong electronegative charge may become bound to the basic groups that arise at cell surfaces as a result of injury. In the experiment previously reported (25) an additional effect *in vivo* of the injection of a strong solution of heparin was seen in the local and temporary resorption of dermal collagen which occurred at the site of injection. As a result, the elasticity of the surrounding skin pulled apart the edges of a wound in such an area and converted the original narrow ellipse into an oval or circular shape. It was thought that the greater mitotic activity necessary to close such wounds might in part account for the localization of tumors to the scars. A later experiment afforded some support for this view (25).

Whether heparin plays any part in tumor formation or growth under normal circumstances is at present unknown. As regards its origin—and largely on the basis of the metachromatic staining reaction—it has been suggested that heparin is discharged into the blood stream from the granules of the histogenous mast cells which line the capillaries of the loose connective tissues (33). In addition to the hematological function of these cells, Sylvén (29, 30) believes that in both normal and malignant growth the histogenous mast cells fulfil a second role in which they migrate away from the capillaries and discharge their granule content into the tissues where it may be detected as metachromatically-staining "free chromotrope substance" (15, 16). If, as Sylvén (30) believes, such "free chromotrope substance" is important in facilitating the invasivenes of tumor cells, many of the observations on the aggregation of mast cells around tumors (2, 11, 13, 21, 28, 32) and in skin which has been subjected to the action of tar (1, 3,7–9, 19, 23, 24, 31), arsenic, X-rays (1) and the carcinogenic hydrocarbons (4, 24) take on a new significance.

It was thus of interest to determine the effect on tumor growth of substances calculated to bind heparin or similar high molecular polysulphuric acid esters in the hope that results of possible therapeutic significance might emerge.

## Preliminary Experiments on Tumor Growth of Heparin-Binding Substances

For the purpose of the present investigation the 2,146 transplantable mouse carcinoma was chiefly employed. During the past 5 years this tumor has been maintained in young inbred (M.R.C. strain) mice by the intramuscular injection every 10 days of 0.25 cc. of a standard suspension of tumor cells in normal saline. By this method 100 per cent "takes" are obtained and the resulting tumors show remarkable uniformity of size during the first 12 to 14 days following transplantation. In consequence, the comparison of the mean size of the tumors in small groups of animals is of value. It is of interest that Stedman, Stedman and Pettigrew (27) in this laboratory used this tumor in experiments in which the addition of basic protein to the suspension of tumor cells at the time of transplantation was found to cause temporary inhibition of tumor

growth. As Chargaff and Olson (5) have shown, protamine is capable of combining with heparin both *in vivo* and *in vitro*.

In the present series of experiments the addition to the normal saline used at the time of transplantation of basic lead acetate (14) or basic metachromatic dyes (20) such as basic fuchsin, brilliant cresyl blue, toluidine blue, neutral violet or acridine red in concentrations varying from 1:100 to 1:5,000 was in each case observed to cause retardation of growth of the transplants. Basic lead acetate and toluidine blue were most active in this respect. The further addition of an excess of heparin to the mixtures of basic dyes and tumor cells prior to transplantation did not overcome the inhibition and such mixtures were toxic. The addition of heparin alone in low concentration was without effect on tumor growth: higher concentrations were inhibitory. In contrast to these results, remote subcutaneous injections each day of 0.5 to 1.0 cc. of the basic compounds mentioned above following routine transplantation of the tumor failed to affect its growth despite the fact that in the case of a dye such as toluidine blue the urine became deeply stained, indicating that the dye had entered the circulation. When, however, following routine transplantation, these dyes were fed to mice in 0.5 per cent concentration in the drinking water, inhibition of tumor growth was obtained with Unna's neutral violet (Gurr). The urine of the mice fed this dye showed a crimson tint; and at autopsy on the tenth day following transplantation the tumors were found to be similarly stained and about one-third the size of the controls. No signs of toxicity were observed in the mice during the period of feeding the dye.

It was therefore decided to investigate the effect of feeding to tumor-bearing mice all the available dyes tested for metachromatism by Lison (20), together with those dyes cited by Herrmann (12) as being capable of combining with heparin *in vitro*.

While the experiment was in progress, the first of an interesting series of papers by Lewis, Sloviter and Goland (17) from the Wistar Institute was noted in which it was stated that the feeding of Nile blue or its oxazone to mice bearing a transplantable sarcoma inhibited the tumor and stained it a diffuse blue. As Nile blue was one of the dyes which had been set aside for use in the present experiment, and as some of the dyes were found to be but sparingly soluble in water, the method of the Wistar group was adopted for the further experiments to be described in detail and the dyes fed in powder form with the food.

### Effect of Feeding Basic Dyes to Mice Bearing a Transplantable Tumor

Of the 55 triphenyl methane, oxazine, thiazine, azine, xanthene and acridine dyes tested by Lison (20) for metachromatism, 34 were obtained from laboratory stocks or from Messrs G. T. Gurr, London. Five dyes of known composition[1] were gifted by the Dyestuffs Division of Imperial Chemical Industries, Ltd., and a sample of Nile blue chloride (hydrochloride of Nile blue base; 3-diethylaminonaphthophenoxazine hydrochloride) was kindly prepared for me by the British Drug Houses, Ltd. To Lison's list was added Janus black which Herrmann (12) found to be effective in precipitating heparin *in vitro*.

From the results of the preliminary experiments it was decided to feed all the dyes in 0.3 per cent concentration in the food. For this purpose each of the 41 dyes in powder form was carefully mixed as required with finely ground Ratcubes in the proportion of 1.5 gm. of dye per 500 gm. of feeding stuff. The dye mixtures were fed from metal hoppers to groups of 5 inbred mice immediately following the transplantation of the 2146 carcinoma; and the controls were fed in exactly similar fashion except that no dye was added to the powdered foodstuff. Water was available throughout.

During the course of the next 10 days specimens of urine from the mice fed dyes were repeatedly obtained on filter paper and the growth rates of the tumors in these animals were compared with those in the controls. Surviving animals were killed on the tenth day following transplantation and were subjected to postmortem examination at which a final estimate of the degree of inhibition of tumor growth was made. Any dye which at the end of 10 days had inhibited the growth of the tumor to less than half that of the controls and which had been found to be unassociated with signs of toxicity to the hosts, was re-tested on a further group of ten animals. The results are presented in Table I and are discussed together with the findings of a comparable experiment in which 8 of the most effective dyes were fed to mice bearing the transplantable sarcoma 180 in place of the 2146 carcinoma herein described.

[1]Magenta P 150 and methyl violet 2 B 200 are capable of forming imino bases; new methylene blue A 125, methylene blue B.P. and Crystal violet B.P.C., in which the amino group is fully substituted, cannot form imino bases.

TABLE I: TABLE OF DYES TESTED FOR METACHROMATISM BY LISON IN 1935 TOGETHER WITH ACTUAL DYES FED IN 0.3 PER CENT CONCENTRATION TO INBRED MICE BEARING THE 2146 TRANSPLANTABLE CARCINOMA

| Dye tested for metachromatism by Lison | Metachromatic color change (Lison) | Actual dye fed | Maker | Toxicity | Size of tumors on 10th day compared with controls | Color of tumor | Color of urine |
|---|---|---|---|---|---|---|---|
| | | **TRIPHENYL METHANES** | | | | | |
| Parafuchsin | Red—Orange | N.A. | — | — | — | — | — |
| Fuchsin | Red—Orange | Basic fuchsin | Gurr | 0 | 1 | — | — |
| | | Magenta P. 150 | I.C.I. | 0 | 1 | — | — |
| Hoffman's violet (Dahlia) | Violet—Red | Hoffman's violet | Gurr | + | 1 | — | Pink |
| Methyl violet R, 2R, 4R | Violet—Red | Methyl violet | Gurr | ++ | 1 | — | — |
| | | Methyl violet 2B.200 | I.C.I. | ++ | ½—¾ | — | — |
| Crystal violet | 0 | Crystal violet | Gurr | +++ | ⅛—¼ | — | — |
| | | Crystal violet B.P.C. | I.C.I. | ++ | ¼—½ | — | — |
| Ethyl violet | 0 | Ethyl violet | B.D.H. | 0 | ¼—½ | Faint blue-green | Buff |
| Methyl green | 0 | Methyl green | Gurr | 0 | ½ | — | — |
| Aniline blue | Blue—Violet | Aniline blue | Gurr | 0 | ¾ | — | — |
| Victoria blue R, B | Blue—Red | Victoria blue B | Gurr | ++ | ¾ | — | — |
| Victoria blue 4R | 0 | Victoria blue 4R | Lust-garten | 0 | 1 | — | — |
| Malachite green | 0 | Malachite green | Gurr | ++ | ¾—1 | — | — |
| Brilliant green extra | 0 | Brilliant green | Gurr | 0 | ⅓ | — | Faint green |
| Sétoglaucine | 0 | Sétoglaucine | Gurr | 0 | ½—¾ | — | — |
| New green solid 2B | 0 | N.A. | — | — | — | — | — |
| Glacier blue | Blue green—Blue violet | Glacier blue | Gurr | 0 | ¾—1 | — | — |
| Sétocyanine O | Blue green—Blue violet | N.A. | — | — | — | — | — |
| Chrome green | 0 | N.A. | — | — | — | — | — |
| | | **OXAZINES** | | | | | |
| Capri blue | 0 | N.A. | — | — | — | — | — |
| Brilliant cresyl blue | Blue—Red | Brilliant cresyl blue | Gurr | 0 | ¾ | — | Blue |
| Oxonine | Blue—Red | N.A. | — | — | — | — | — |
| Meldola'a blue (New blue R) | 0 | Meldola's blue | Gurr | 0 | 1 | — | — |
| New blue B | Blue—Violet | N.A. | — | — | — | — | — |
| Nile blue | Blue—Violet | Nile blue | Gurr | 0 | ¼ | Blue | Blue |
| | | Nile blue sulphate | ? | ++ | ¼—½ | Blue | Blue |
| | | Nile blue chloride | B.D.H. | 0 | ⅛—¼ | Blue-green | Purple |
| | | **THIAZINES** | | | | | |
| Thionine (Lauth's violet) | Violet—Red | Thionine | Gurr | ++ | ½—¾ | — | — |
| Toluidine blue | Blue—Red | Toluidine blue | Gurr | 0 | ½—¾ | — | Deep blue |
| Methylene Azures A, B, C | Blue—Red | Azure I | Gurr | 0 | ½—¾ | — | Blue |
| Methylene blue | 0 | Methylene blue (Bact.) | Gurr | ++ | ¾—1 | — | Blue |
| | | Methylene blue B.P. | I.C.I. | + | ¾—1 | — | Blue |
| Methylene green | 0 | Methylene green | Gurr | 0 | 1 | — | Blue green |
| New methylene blue | Blue—Violet | New methylene blue | Gurr | + | ½—¾ | — | Blue |
| | | New methylene blue A. 125 | I.C.I. | 0 | 1 | — | Deep blue |
| | | **AZINES** | | | | | |
| Neutral red extra | Red—Yellow | Neutral red | Gurr | + | 1/12—¼ | Red | Red |
| Neutral violet extra | Violet—Red | Neutral violet | Gurr | 0 | ⅛—¼ | Crimson | Crimson |
| Bâle blue R | 0 | N.A. | — | — | — | — | — |
| Safranine T | Red—Yellow | Safranine O | Gurr | 0 | ½—¾ | — | Orange |
| Indazine M | Blue—Red | N.A. | — | — | — | — | — |
| Janus blue (Naphtindone BB.) | Blue—Violet | Janus blue (alcoholic) | Gurr | 0 | ¾ | — | — |
| Janus green | Green—Red blue | Janus green B | Gurr | +++ | ⅛—¼ | — | — |
| | | Janus black | ? | ++ | ¼—½ | — | — |
| Heliotrope 2B | 0 | N.A. | — | — | — | — | — |
| Heliotrope of tannin | Red violet—crimson | N.A. | — | — | — | — | — |
| Magdala red | Red—Red | Magdala red | Gurr | 0 | ¾—1 | — | — |
| | | **XANTHENES AND ACRIDINES** | | | | | |
| Pyronin G | 0 | Pyronine | Gurr | 0 | 1 | — | — |
| Acridine red 3B | Red—Yellow | Acridine red | Gurr | 0 | ¾ | — | Pink |
| Rhodamine S, B, 3B G, 3G, 6G | 0 | Rhodamine | Gurr | 0 | ¾ | Orange | Orange red |
| Acridine yellow | Yellow—? | Acridine yellow | Gurr | + | ½—¾ | — | Yellow |
| Acridine orange | 0 | Acridine orange | Gurr | 0 | ¾—1 | Faint yellow | Yellow |
| Trypaflavine | 0 | Acriflavine | B.D.H. | ++ | ½—¾ | — | Yellow |

"N.A." = dye not available.

RESULTS AND DISCUSSION

As the accompanying table indicates, those dyes which Herrmann (12) found to be effective in his studies on blood coagulation *in vitro* (Janus green, Janus black, crystal violet, methylene blue, methyl green) all inhibited the growth of the tumor when fed in 0.3 per cent concentration to mice bearing the 2146 carcinoma. Yet their inhibitory powers were paralleled by their toxicity and no staining of the tumor with these dyes was observed. On the other hand, *in vivo* staining and significant inhibition of growth of the 2146 carcinoma without undue signs of toxicity to the hosts were obtained by feeding neutral violet, neutral red and Nile blue in similar concentration.[2] Slight inhibition of growth and faint staining of the tumors were also observed in the mice fed ethyl violet.

In the case of neutral violet, 12 of 15 mice survived 10 days and at autopsy the tumors were found to be stained a diffuse crimson, their sizes ranging from 1/8 to 1/4 that of the controls. No other tissue appeared to be as selectively stained as the tumor, although the liver in such mice was often somewhat friable and of a light chocolate color. Sections of liver from these mice showed a mild zonal necrosis. Apart from their small size and unusual color, the tumors appeared healthy and, in fresh preparations, no particular cytological location of the dye could be detected. Similar, though more marked, changes in tumor color and liver histology were noted when neutral red was employed, and this dye was more toxic than neutral violet. On inquiry to the maker it was found that Unna's neutral violet (Gurr) is a mixture of Neutral red and New blue (Meldola's blue): and since Meldola's blue alone failed to stain or inhibit the tumor, it is evident that the inhibitory effect of Unna's neutral violet is due to its content of neutral red.

Nile blue (Gurr), Nile blue sulphate (maker unknown) and Nile blue chloride (British Drug House) were found to yield the results already described by the American workers. Of the original 15 mice in each of the three groups that received these dyes, 11, 7, and 13 animals respectively survived 10 days. Toxicity was least marked in the mice receiving Nile blue chloride (B.D.H.) and this dye stained the tumors a greener shade than did the other Nile blues. In small carcinomas the whole tumor stained diffusely. In larger tumors, or if the staining was faint, often only a narrow rim around the periphery of the growth assumed the color. As in the case of neutral violet or neutral red it was not possible to detect selective staining of any particular structure in the tumor cells. Liver damage was not seen in the mice fed Nile blue and Nile blue chloride: though slight toxic changes were noted in the livers of mice fed Nile blue sulphate. In addition, the upper pole of the testis and adjacent epididymis, thymus, submaxillary salivary glands and an occasional lymph node were, like the tumor, frequently seen to be diffusely stained though it was rare to observe all these structures staining in the same animal: nor was the color in these organs as intense as in the tumor itself.

Since the publication of their first paper (17), Lewis, Sloviter and Goland (18) have recorded *in vivo* staining and inhibition of growth of their transplantable sarcoma in mice fed Cresyl violet, an oxazine dye closely related to Nile blue; and they have also obtained comparable results as regards inhibition and staining with some of the xanthene and acridine dyes. Specimens of 3 of these dyes (acridine red, acridine orange and rhodamine) were among those employed in the present experiment: and while faint diffuse staining of the 2146 carcinoma was observed with acridine orange (Gurr) and rhodamine (Gurr), their selective action appeared to be less marked than in the case of neutral violet, neutral red and Nile blue. The resultant inhibition of tumor growth with all 3 dyes was negligible.

In view of the fact that the American workers used mainly a transplantable sarcoma in their experiments, it was anticipated that the results of the present investigation might become more striking when the transplantable mouse sarcoma 180 was employed. Such was not the case. The inhibition of this tumor with the three specimens of Nile blue was slight and the staining of the tumor was invariably less than that of testis, thymus or salivary gland. Even neutral red in 0.5 per cent concentration in the food produced an insignificant reduction in tumor size, though the tumor itself stained a deep crimson. Similarly, ethyl violet in 0.5 per cent concentration stained this tumor a diffuse blue-green

[2] In so far as statistical analysis of such small groups permits, the following figures may be of interest.

| Tumor | Number measured | Mean diameter on 10th day, mm. | Standard deviation |
|---|---|---|---|
| Controls | 50 | 9.98 | 1.63 |
| Neutral violet | 12 | 4.42 | 1.09 |
| Neutral red | 10 | 3.40 | 0.96 |
| Nile blue | 11 | 5.09 | 0.70 |
| Nile blue sulphate | 7 | 6.00 | 0.82 |
| Nile blue chloride | 13 | 4.00 | 0.76 |

In each case the probability of the dye-fed and control tumors being samples from the same universe is less than 0.01.

shade but failed to retard its growth. It must be concluded that the American workers were fortunate in their choice of tumor on which the effects of feeding most of the dyes in the Color Index were carefully scrutinized. In view of the theoretical considerations which prompted the present investigation, it would have been of great interest to have repeated this experiment on the 3187 transplantable mast cell sarcoma. Unfortunately this tumor is no longer available.

Nevertheless, the combined results of this and the American investigation clearly indicate that of the great number of dyes tested, only a few have so far been found to cause selective *in vivo* staining and inhibition of tumor growth when fed to mice. Of this few it is no doubt of significance that all are to be found in those groups of dyes which Lison (1935) tested for metachromatism. On the other hand, not all dyes which are metachromatic are capable of staining tumors and inhibiting their growth when fed to mice: nor do toxicity and inhibitory power run parallel. The fact that it is necessary to feed the dyes before subsequent staining and inhibition are obtained suggests that the dyes undergo metabolic alteration before they reach the tumor. Further work is required before the underlying mechanisms become clear.

## SUMMARY

Addition of basic lead acetate or basic metachromatic dyes to the suspension of the cells of the 2146 transplantable mouse carcinoma prior to its inoculation into inbred mice resulted in retardation of growth of the transplants. In contrast, repeated remote subcutaneous injections of these compounds into mice following routine transplantation of the tumor failed to affect its subsequent growth. When, however, 41 basic dyes in 0.3 per cent concentration in powdered ratcubes were fed to mice bearing the 2146 carcinoma, neutral violet and neutral red were found to inhibit the growth of the tumor and stained it crimson. Faint blue-green staining and slight inhibition of the tumor were also obtained with ethyl violet. As has recently been reported elsewhere, the feeding of Nile blue was similarly found to cause inhibition of tumor growth and to stain the carcinoma a diffuse blue. The inhibition of growth and *in vivo* staining by these dyes were much reduced when the transplantable mouse sarcoma 180 was employed. The results of this and similar experiments are briefly discussed.

## ACKNOWLEDGMENTS

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# In Memoriam: George Frederick Laidlaw (1871-1937)

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George Frederick Laidlaw, the man in whose memory your president has asked me to speak, was in many respects an uncommon individual. You will realize this when I tell you that all of the effective research which he accomplished was done during the last ten of his sixty-six years of life. It is indeed only the exceptional man who, after a long and busy career, can retire from the occupation of a lifetime and engage in investigation in an unrelated field with such success that his accomplishments therein form permanent contributions to the body of knowledge.

He was born June 26, 1871, in Jersey City and graduated from the New York Homeopathic College in 1890. Although he had a great interest in pathology at this time, he could obtain no position which would enable him to pursue this subject and pay enough to support himself and his family and so he turned to the general practice of medicine. In this he was successful. He developed a good practice and later specialized in cardiology. During these years he did not allow his mind to lie fallow. Some twenty publications show that he was thinking about the problems raised by his patients and that he was able to make suggestions for answering them. The subjects dealt with are various but concern chiefly tuberculosis and cardiovascular disease.

About 1920, when he was forty-nine years old, an event occurred which was to change the whole scope and tenor of his life. He discovered that he had papillary growths in his bladder. During the next few years he was fulgurated several times and underwent two operations. This forced him to withdraw from active practice. He still had an interest in cardiology and occupied some of this time translating from the French, Vaquez "Diseases of the Heart," which he regarded as the finest work on the subject. This was published by Saunders in 1924.

But when this was done he determined to devote the rest of his life to the study of cancer since he himself had been attacked by it. His familial and financial responsibilities were lessened so that he could devote all of his energies to this new undertaking. His first step was to visit a number of different laboratories abroad and learn what he could from their directors. He spent time with Cajal, Nageotte, Roussy and many others and finally came to the conclusion that Pierre Masson, the French pathologist of Strasbourg, was the master in histopathology and, as he always sought the best in everything, he determined to study with Masson. The story of the relationship of these two men is graphically recounted by Masson in a letter which I shall read to you shortly. Suffice it to say now that Laidlaw did study with Masson when the latter first came to Montreal and absorbed the details of his technical procedures and his conceptions of nevi, glomus tumors, appendiceal neuromas and other peripheral nervous structures. As a result of his work with Masson and his visits to other laboratories he came to the conclusion that the relationship between nervous activity and cancer deserved more investigation and he wanted to begin by studying the skin lesions and tumors of von Recklinghausen's disease.

He was eager to discover some laboratory where he might find a place to work and material to work with. This led him to Wilder Penfield who had recently worked with del Rio Hortega and had established a laboratory of neurocytology at the Presbyterian Hospital, in New York. Penfield said that if he was going to push the study of neurofibromas it would be necessary to have a really adequate connective tissue stain that would demonstrate reticulin fibers well and suggested that he try to develop one. This work was carried on throughout the latter half of 1927 and the beginning of 1928 in Penfield's laboratory and through 1928 in the Columbia University Laboratory of Surgical Pathology to which he allied himself when Penfield went to Montreal. The result of this investigation was published in May 1929, in the *American Journal of Pathology,* and the *Comptes Rendus de la Société de biologie* in France and is now spoken of as

* The Editors regard this Memorial of sufficient interest to have it published even at this late date.

the Laidlaw stain. Not only is it an excellent method of staining collagen and reticulin but the sharp eyes of Laidlaw noticed that after formol or Bouin fixation epithelial cells were stained, whereas those of connective tissues were not. It was hoped that this might serve to distinguish these tissues one from the other not only under normal circumstances but also in tumors. Further experience with the stain has shown, however, that there are too many variations with undifferentiated tumor cells to make it a reliable method.

Laidlaw next used this stain to demonstrate the endoneurial sheath of reticulin fibers which forms a meshwork about the cerebral and spinal nerves beginning at the exact point where they emerge from the brain and cord. This sheath was first described by Plenk in 1927, but Laidlaw's demonstration of its exact structural composition has led some writers to refer to it as the Plenk-Laidlaw sheath. This work was published in the same two American and French journals in 1930.

Laidlaw next turned his attention to melanin. During 1932, 1933 and 1934, a series of papers were published dealing with Bloch's dopa reaction for which he worked out a simplified and reliable technic and with the distribution of melanin in health and disease, and culminating in his theory of pigmented moles. He came to the conclusion that "the pigmented hairy mole appears to be a link or transition from pigmented tactile organs of the reptilian type to hairy tactile organs of the mammalian type. In its hair follicles it is mammalian, in its pigmentation, elevation and in the groups of innervated tactile cells in the corium it follows the amphibian-reptilian pattern." This work necessitated the staining of axis cylinders and he did an enormous amount of experimental work trying to develop a reliable technic for use after ordinary fixatives and paraffin embedding. He modified the Gros and the Rogers technics, as well as many others, but never achieved consistent success. The only entirely reliable method in use in our laboratory at the present time is the modification of Cajal's impregnation method after chloral hydrate fixation, which was developed by Dr. and Mrs. Laidlaw and has never been published.

His last work concerned itself with the granules in the islet cells of the pancreas. It happened that a whole series of islet cell tumors were removed by Whipple during 1934 and 1935, and his interest was aroused in trying to develop a reliable differential stain for them. He tried all of the different methods with many modifications but was never satisfied with the results. He left behind him an almost completed manuscript on the histopathology of these tumors which Mrs. Laidlaw and I have prepared for publication.

Late in 1935, the tumor which had remained localized for so many years finally manifested itself in his pelvic bones. It was obvious to all of us that it was the beginning of the end but he never accepted it. He would never admit to himself that this was the spread of a cancer but continued his work and laid plans for future work until he was no longer able to remain upright. Even when confined to bed his mind continued its activity until he died on June 22, 1937.

George Laidlaw worked beside me in the Surgical Pathology Laboratory of Columbia University for the last nine years of his life. No young man with the vigor and energy of youth ever surpassed him in devotion, enthusiasm or productivity. He was a constant stimulant to his associates, a pleasant and inspiring companion, a valued friend. His place in our laboratory cannot be filled.

I shall close this memorial notice with two quotations: Writing to me about Dr. Laidlaw, Wilder Penfield said: "I shall never again say that a man is too old to take up new work. . . After knowing Laidlaw it seems to me that the only disadvantage in starting a new technical problem as one approaches sixty is that his life span is too short. I am sorry that Laidlaw could not have had fifty years in which to satisfy his enthusiastic craving for new knowledge." The second quotation is the following letter received from Professor Pierre Masson in response to a request that he write something which I might read at this meeting:

(Translation from the French approved by Professor Masson).

November 16, 1937

My dear Colleague:

Our friend, G. F. Laidlaw, has held such a large place in my life these past ten years that I gladly accept your invitation.

He was a well rounded man, and there is no better way of portraying him than to tell the story of his relations with me.

In the middle of December, 1926, at the moment when I was leaving the Pathological Institute at Strasbourg for the last time, an unknown man passed me at the threshold and, addressing himself to the doorkeeper, made known to him his wish to see me. I turned around and told him I was the person he sought. Then the following conversation took place:

"Ah, you are Professor Masson. I am George

Laidlaw, and I have come to ask you for a place in your laboratory."

"You come at an unfortunate moment," I replied; "I am leaving for Paris and Montreal."

"For how long?"

"I don't know, for years probably. But you are welcome to stay here. My substitute will certainly give you the place you want."

"Not at all," he said to me. "It is with you that I wish to work. Since you are going to Montreal, I will go there also if you can find a place for me."

A little surprised—one would be that at least—I put several questions to Laidlaw. "But why are you so anxious to work with me? There are other pathologists."

Then, briefly, Laidlaw told me his story. "I am a practicing physician in New York or, rather, I was. I was even a specialist in cardiology; I am the one who translated the treatise of Vaquez into English. One month ago I learned I had a cancer of the bladder. I said to myself: 'Since I must die of cancer, I want to spend my last days investigating cancer.' I have given up my practice. I have read your book on Tumors and your article about the Naevi. I have decided to study the Naevi with you. I had my bladder fulgurated. I took the first boat, and here I am. But since you are leaving, I will rejoin you in a month in Montreal."

"Very well," I replied, "I'll see you then."

The whole conversation did not last five minutes and took place on the sidewalk in front of the Pathological Institute at Strasbourg. We parted after a vigorous handshake.

A fortnight afterwards I was beginning to get settled in Montreal. Upon his return to New York Laidlaw wrote to ask me if he might come at the agreed time. Upon receiving my reply in the affirmative, he arrived, bringing a superb microscope, and he remained five months in my laboratory.

He was a beginner, and I confess that his intention of introducing himself to the histology of tumors by beginning with the study of the Naevi both astonished and disturbed me. My astonishment soon changed into admiration.

At this time Laidlaw was long past his fiftieth year. Never have I encountered a younger or more comprehensive mind. Arising at five o'clock in the morning, he read until eight. At nine o'clock he came to the laboratory and did not leave it until six o'clock in the evening.

Not only did he study my sections of Naevi but he understood them. He began to acquire technique and learned normal and pathological histology with a disconcerting rapidity and exactness.

Never have I seen such a hunger to learn.

We talked of nothing but tumors. Laidlaw was not only an enthusiastic beginner in histology. He was a remarkable physician, a scholar, a man of letters. The French and German classics had no secrets for him. Goethe was his favorite author. I really believe that he knew 'Faust' by heart. He quoted long passages from it on appropriate occasions.

And he was above all a friend. Many times he came to see me, many times he welcomed me in New York. He had given me his friendship and smilingly called himself 'Masson's Ambassador to the United States'. Not only did he introduce me to his friends among the pathologists of New York, but he himself translated the papers which I sent to American journals and he did it with such care and devotion that he never made a single mistake. If my American colleagues know me, it is to him that I owe it.

Laidlaw was my friend, a genuine, affectionate, disinterested friend. His feelings for me have always filled me with legitimate pride and profound gratitude.

In 1926, believing himself condemned, Laidlaw suddenly changed the whole orientation of his career. Death waited eleven years for him. During this respite, reverses and troubles assailed him without disturbing his optimism and his love for Science. During the last months of his life, when his illness, reawakened, definitely confined him to his bed, he wrote to me still and finally had Mrs. Laidlaw write me letters full of courageous serenity.

During these eleven years he made a name for himself among the histopathologists. There are Laidlaw techniques which are used in all laboratories. There are articles by Laidlaw which everyone cites.

All that will endure. But what would have been the reputation of this man if he had begun his true career at an earlier age, the career for which he was made and to which he only devoted the final one sixth of his life!

I stop because I perceive that no doubt this story is too long for your purposes. But you asked me for some notes about my friend Laidlaw, he who was fine, intelligent, scholarly, filled with curiosity, determined, full of humor, and finally stoically courageous: the perfect American who has made me know and love America, and so my pen began to run along . . .

Cordially yours,

P. Masson

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